Docket No.: 218874US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

Michel RENARD, et al. : EXAMINER : BAUM, S.F.

SERIAL NO: 10/030,194 :

FILED: AUGUST 15, 2002 : ART UNIT: 1638

FOR: MUTANT GENE OF THE GRAS FAMILY AND PLANTS WITH REDUCED DEVELOPMENT CONTAINING SAID MUTANT GENE

APPEAL BRIEF

COMMISSIONER FOR PATENTS
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SIR:

This is an appeal of Claims 1, 4-8, and 11-16 in the above-identified application and the rejections set forth in the final Official Action mailed October 5, 2007.

I. Real Party of Interest

The real party of interest is Institut National De La Recherche Agronomique (INRA), by virtue of the assignment recorded in the U.S. Patent and Trademark Office on November 5, 2002, at reel 013460, frames 0736-0739.

II. Related Appeals and Interferences

Appellants, Appellants' legal representative and their assignee are not aware of any appeals or interferences which will directly affect or be directly affected by or having a bearing on the Board's decision in this appeal.

III. Status of Claims

Claims 1, 4-8, and 11-16 are the only claims pending in the above-identified application and appear in the attached Claims Appendix. All other claims, whether original or added during prosecution, were canceled during prosecution of this application.

Claims 1, 4-8, and 11-16 stand rejected.

Claims 1, 4-8, and 11-16 are appealed herein.

IV. Status of Amendments filed under 37 C.F.R. §1.116

An Amendment under 37 C.F.R. §1.116 was not filed. Appellants now appeal the rejections set forth in the final Office Action mailed October 5, 2007.

V. Summary of the Claimed Subject Matter

As recited in independent Claim 40, the present invention provides an isolated nucleic acid sequence obtained by mutation of a sequence encoding a plant protein of the GRAS family, the wild-type form of which comprises the following peptide sequence (SEQ ID NO:5):

Gly Tyr X_1 Val Glu Glu

in which X_1 represents arginine or asparagine, wherein said mutation results in a modification of said sequence (I, SEQ ID NO:5) such that the nucleic acid sequence encodes a mutant protein comprising the following peptide sequence (SEQ ID NO: 7):

Gly Tyr X_1 Val Glu X_2

in which X_1 is as defined above, and X_2 represents a basic amino acid, and

wherein a plant transformed with said isolated nucleic acid, which expresses said mutant protein exhibits a reduction in plant size as compared to the wild-type plant (see the specification at page 3, line 30 to page 5, line 25, see in particular page 4, lines 31 to page 5, line 9, and page 6, line 26 to page 7, line 19).

The present invention also provides plants with reduced development, mutant plants with reduced development, and descendent plants thereof, which contain one or more copies of a nucleic acid sequence defined above (see the specification at page 5, line 34 to page 7, line 19).

VI. Grounds of Rejection to be Reviewed on Appeal

1. Claims 1, 4-8, and 11-16 stand rejected under 35 U.S.C. §112, first paragraph (enablement).

2. Claims 5-8, 11, and 13-16 stand rejected under 35 U.S.C. §102(b) over Foisset et al[1] taken with the evidence of Barret et al[2].

[1] Foisset et al, *Theor. Appl.* Genet., 91(5): 756-761 (1995).
[2] Barret et al, *Theor. Appl. Genet.*, 97: 828-833 (1998).

VII. Arguments

(A) Claims 1, 4-8, and 11-16 stand rejected under 35 U.S.C. §112, first paragraph, as lacking enablement. This rejection is untenable and should not be sustained.

The Examiner appears to recognize that the specification is enabling for a mutant plant in the family Brassicaceae, obtained by chemical mutagenesis, and comprising a mutant gene encoding a protein comprising the amino acid sequence SEQ ID NO: 7. However, the Examiner alleges that the specification fails to enable an isolated mutant gene encoding a protein comprising the amino acid sequence GYX_1VEX_2, wherein X_1 is R or N and X_2 is a basic amino acid, wherein the sequence is SEQ ID NO: 7 or 4, or for transgenic plants transformed with said gene.

Appellants respectfully disagree with the Examiner's allegations with respect to a purported lack of enablement. To this end, Appellants submit that the information provided by the specification combined with the prior art knowledge is sufficient to allow one of skill in the art to produce and isolate the mutant sequence.

In the specification, it is disclosed that the mutant gene is derived from a sequence encoding a protein of the RGA/GAI subgroup of the GRAS family, that the wild-type protein must comprise the sequence GYRVEE or GYNVEE, and that the claimed mutation results in the substitution of the C-terminal "E" of this sequence with R or K. The specification further provides the full-length sequences of wild-type and mutant cDNAs and proteins.

Appellants submit that the GRAS family is a well known family of proteins and that members of this family would be readily apparent to the skilled artisan. Appellants submit that the "GRAS family" (also known as the VHIID family) was already known at the time of the present invention. Further, this family is characterized by several conserved motifs. To demonstrate the state of the art that existed at the time of the present invention Appellants

refer to two references discussed on pages 2-3 of the present application as they relate to the description of the GRAS family. These references are:

1) Pysh et al, *The Plant Journal* (1999) **18**(1), 111-119; and

2) Schumacher et al, *Proc. Natl. Adad. Sci. USA* (1999) **96**, 290-295.

From the foregoing, Appellants submit that at the time of the present invention, the GRAS family and the RGA/GAI subfamily (DELLA family) were known in the art. Moreover, conserved sequence motifs allowing the recognition of whether a protein belongs to the GRAS family, and more specifically to the RGA/GAI subfamily were clearly defined. To further support this position, attention is directed to the publication of Peng et al. (1999) (cited by the Examiner in the Office Action mailed May 8, 2006) and the publication of Silverstone et al. (1998) (Annex 1 to the response filed on November 8, 2006). Thus, it would have been only a matter of routine experimentation to query the sequence databases with the full-length sequences disclosed in the present specification, to identify those corresponding to proteins of the RGA/GAI subfamily having a GYRVEE or GYNVEE sequence, and to isolate the corresponding gene.

Alternatively, one of skill in the art would also have been able to design, from the highly conserved sequences within the RGA/GAI subfamily and from the sequence information provided by the present specification, appropriate probes and/or primers allowing isolation of a cDNA encoding a protein belonging to the RGA/GAI subfamily and comprising the sequence GYRVEE or GYNVEE from a cDNA library of a plant without undue experimentation. Once the wild-type cDNA is isolated, it is also only a matter of routine experimentation to perform directed mutagenesis in order to replace the codon for "E" with a codon for "R" or "K".

Further, once the mutant sequence is obtained, inserting it in an appropriate construct and performing plant transformation is also well within the ordinary skill in the art. The Examiner's comments referring to the unpredictable results which are obtained when transforming plants with genes that are involved in plant development are not relevant, since they refer to a state of the art concerning genes that do not encode proteins of the RGA/GAI family, and that are not even related to the GRAS family. REB is a basic leucine-zipper (bZIP) transcription factor, OSH1 is an homeobox transcription factor, and CBFs belong to the AP2/EREBP family.

The present invention concerns a mutant transcription factor of the RGA/GAI family. Thus, the prior art to be considered should relate to transgenic plants expressing mutant transcription factors of the RGA/GAI family. Peng et al. (1999) disclose several mutants of RGA/GAI transcription factors that are semi-dominant mutations that confer a dwarf, giberellin-resistant phenotype. Although these mutants do not involve the same part of the protein, these mutations disclosed by Peng et al. are functionally similar to the mutation disclosed in the present application, i.e. they are semi-dominant mutations that confer a dwarf, giberellin-resistant phenotype. Peng et al. further disclose (see page 261 "Rice transformants", and Figure 4) the production of transgenic rice expressing a mutant gene of Arabidopsis, under control of the ubiquitin maize promoter (which is a well-known constitutive and ubiquitous promoter). All the plants containing the transgene have a dwarf giberellin-resistant phenotype.

It clearly appears from the prior art that transformation of plants by a gene encoding a mutant results in transgenic plants having the desired phenotype, without having to chose a promoter providing a specific pattern of expression, and without performing screening steps other than the classical detection of the transgene. Thus, there is no reason to presume that

replacing the RGA/GAI mutant gene of Peng et al. by the RGA/GAI mutant gene of the invention will not result in the production, in the same way, transgenic plants having the desired phenotype.

Therefore, in view of the information provided by the specification combined with the prior art knowledge one of skill in the art would have been able to practice the claimed invention without undue experimentation.

Accordingly, it is respectfully requested that this rejection be REVERSED.

(B) Claims 5-8, 11, and 13-16 stand rejected under 35 U.S.C. §102(b) as being anticipated by Foisset et al taken with the evidence of Barret et al. This rejection is untenable and should not be sustained.

The Examiner alleges that Foisset et al anticipate the present invention because this publication is cited in the specification as reporting the existence of a plant having the *bzh* gene that is responsible for the mutant phenotype. However, the Examiner has not explained how Foisset et al provide an enabling disclosure of this mutant plant, *i.e.* a disclosure that combined with knowledge in the prior art, would allow one of ordinary skill in the art to grow and cultivate the plant (MPEP 2121.03).

Actually, Foisset et al disclose that the mutation results from with EMS. Following this teaching, one of skill in the art is able to perform chemical mutagenesis of seeds, to grow all the plants from the mutagenized seeds and to select the plants that have a reduced development. He is likely to obtain many plants having a reduced development, since, as already explained in the response to the previous Office Actions, many genes have been identified as involved in dwarfism (and probably, many genes not yet identified are also involved). The Examiner is reminded that EMS mutagenesis is non-discriminatory. EMS

mutagenesis primarily induces G→A substitutions. Therefore, the only suggestion that the skilled artisan would take from Foisset et al is that the *bzh* mutation is likely a G→A substitution. However, the size of the rapeseed genome is about 1200 x 10^6 bp. If one considers a G/C content of approximately 50%, there would be about 600 x 10^6 possible G→A substitutions genome-wide. Clearly, the disclosure of Foisset et al would not place the skilled artisan in of the specific *bzh* mutant plant of the present invention; much less provide information on the gene involved in the *bzh* mutation or the position of the mutation within this gene.

Barret et al further disclose that the *bzh* mutation is semi-dominant. In order to determine if one or more of the EMS mutants selected on the basis of their reduced development has a semi-dominant mutation, the skilled artisan would have to study the progeny of each of these mutants by performing appropriate crosses to obtain homozygous and heterozygous plants for each of the mutation in order to compare them. If the mutation is semi-dominant the homozygous plants should be dwarf, while the heterozygous plants should be semi-dwarf. This will involve clearly a great amount of experimentation, in particular in view of the fact that it may be difficult to distinguish the homozygous dwarf plants from the heterozygous semi-dwarf ones, due to the influence of both the genetic background and the environment on the expression of this character, as indicated by Barret et al (page 828, 2"d column 1st paragraph).

Further, even if one succeeds at identifying plants having a semi-dominant mutation, he will still not be able to determine whether or not there is among them a plant with the *bzh* mutation, since he will have no means to detect this particular mutation and thus to differentiate it from other mutants having a similar phenotype. Thus, he will not be in possession of the bzh mutant plant reported by Foisset et al.

In the Office Action mailed October 5, 2007, the Examiner merely contends that the pending claims "are drawn to plants comprising the mutant nucleic acid sequence of claim 1, which read on the plants of Foisset et al". Citing *Atlas Powder Co.* v. Ireco Inc., 190 F.3d 1342, 1347, 51 USPQ2d 1943, 1947 (Fed. Cir. 1999), the Examiner further alleges that "the claiming of a new use, new function or unknown property which is inherently present in the prior art does not necessarily make the claim patentable."

Appellants disagree with the Examiner's unsupported conclusion of inherency. Indeed, "[t]he fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993); *In re Oelrich*, 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981). "In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy,* 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) In this case, the Examiner has clearly failed to meet this burden.

Further, as discussed above, EMS mutagenesis is non-discriminatory. EMS mutagenesis primarily induces G→A substitutions. Therefore, the only suggestion that the skilled artisan would take from Foisset et al is that the *bzh* mutation is likely a G→A substitution. However, the size of the rapeseed genome is about 1200 x 10^6 bp. If one considers a G/C content of approximately 50%, there would be about 600 x 10^6 possible G→A substitutions genome-wide. Again, the disclosure of Foisset et al would not place the skilled artisan in of the specific *bzh* mutant plant of the present invention, much less provide information on the gene involved in the *bzh* mutation or the position of the mutation within this gene. Put simply, the skilled artisan would have no means to determine whether or not

one of the plants selected after EMS mutagenesis has the same *bzh* mutation as in the presently claimed invention.

Accordingly, it is respectfully requested that this rejection be REVERSED.

VIII. CONCLUSION

For the above reasons, Claims 1, 4-8, and 11-16 are not unpatentable as lacking enablement or as being anticipated by Foisset et al taken with the evidence of Barret et al. Therefore, the Examiner's rejections should be REVERSED.

Respectfully submitted,

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Attachments: Claims Appendix: Pending Claims in U.S. Application Serial No. 10/030,194
Evidence Appendix
Related Proceedings Appendix

CLAIMS APPENDIX

Pending Claims in U.S. Application Serial No. 10/030,194

1. An isolated nucleic acid sequence obtained by mutation of a sequence encoding a plant protein of the GRAS family, the wild-type form of which comprises the following peptide sequence (SEQ ID NO:5):

Gly Tyr X_1 Val Glu Glu

in which X_1 represents arginine or asparagine, wherein said mutation results in a modification of said sequence (I, SEQ ID NO:5) such that the nucleic acid sequence encodes a mutant protein comprising the following peptide sequence (SEQ ID NO: 7):

Gly Tyr X_1 Val Glu X_2

in which X_1 is as defined above, and X_2 represents a basic amino acid, and

wherein a plant transformed with said isolated nucleic acid, which expresses said mutant protein exhibits a reduction in plant size as compared to the wild-type plant.

2. – 3. (Canceled)

4. The nucleic acid sequence as claimed in claim 1, wherein it encodes the polypeptide represented by SEQ ID NO: 4.

5. A plant with reduced development, comprising one or more copies of a nucleic acid sequence as claimed in claim 1.

6. The plant as claimed in claim 5, wherein it is crucifer.

7. The plant as claimed in claim 5, wherein it is a member of the family Brassicaceae.

8. The plant as claimed in claim 7, chosen from rapeseed, cabbage, turnip, brown mustard and Ethiopian mustard.

9. – 10. (Canceled)

11. A plant with reduced development, comprising one or more copies of a nucleic acid sequence as claimed in claim 4.

12. The nucleic acid sequence as claimed in claim 1, wherein X_2 is a lysine.

13. A plant with reduced development, comprising one or more copies of a nucleic acid sequence as claimed in claim 12.

14. A mutant plant with reduced development, wherein said mutant plant is obtained by chemical mutagenesis and comprises one or more copies of a nucleic acid sequence of claim 1.

15. The mutant plant of claim 14, wherein said mutant plant is a rapeseed plant.

16. (A descendant of the mutant plant of claim 14, comprising one or more copies of said nucleic acid sequence.

EVIDENCE APPENDIX

1. Pysh et al, *The Plant Journal* (1999) **18**(1), 111-119; and

2. Schumacher et al, *Proc. Natl. Adad. Sci. USA* (1999) **96**, 290-295.

3. Peng et al., *Nature*, 400:256-261 (1999) (cited by the Examiner in the Office Action mailed July 29, 2005),

4. Silverstone et al., *The Plant Cell*, 10: 155-169 (1998) (submitted as Annex 1 to the response filed on November 8, 2006)

The Plant Journal (1999) 18(1), 111–119

SHORT COMMUNICATION

The GRAS gene family in Arabidopsis: sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes

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Summary

Mutations at the *SCARECROW* (*SCR*) locus in *Arabidopsis thaliana* result in defective radial patterning in the root and shoot. The *SCR* gene product contains sequences which suggest that it is a transcription factor. A number of Arabidopsis Expressed Sequence Tags (ESTs) have been identified that encode gene products bearing remarkable similarity to *SCR* throughout their carboxyl-termini, indicating that SCR is the prototype of a novel gene family. These ESTs have been designated *SCARECROW-LIKE* (*SCL*). The gene products of the *GIBBERELLIN-INSENSITIVE* (*GAI*) and the *REPRESSOR of ga1–3* (*RGA*) loci show high structural and sequence similarity to *SCR* and the *SCLs*. Sequence analysis of the products of the GRAS (*GAI, RGA, SCR*) gene family indicates that they share a variable amino-terminus and a highly conserved carboxyl-terminus that contains five recognizable motifs. The *SCLs* have distinct patterns of expression, but all of those analyzed show expression in the root. One of them, *SCL3*, has a tissue-specific pattern of expression in the root similar to *SCR*. The importance of the GRAS gene family in plant biology has been established by the functional analyses of *SCR*, *GAI* and *RGA*.

Introduction

Identification of gene families is an important first step in elucidating the common molecular mechanisms by which members of the family function and in establishing the biochemical structures and interactions responsible for their activities. Sequence information is routinely used to identify specific functional domains. Sequence comparisons can also identify residues potentially vital for the function of the gene products, based on their absolute conservation in all members of the family. The effects of mutations at these sites may then be determined through reverse genetics.

We report the molecular analysis of a novel plant gene family in *Arabidopsis thaliana*. The first member of this family, *SCARECROW* (*SCR*), was isolated as the result of a screen for mutations that affect root development (Benfey *et al.*, 1993). Mutations at the *SCR* locus disrupt radial patterning of the root, resulting in the loss of a layer of ground tissue (Scheres *et al.*, 1995). The predicted *SCR* gene product contains a number of putative domains which strongly suggest that SCR functions as a transcription factor (Di Laurenzio *et al.*, 1996). A comparison of the predicted SCR sequence with sequences present in the databases revealed that several Arabidopsis Expressed Sequence Tags (ESTs) encode gene products with homology to a region termed the VHIID domain (Di Laurenzio *et al.*, 1996). Subsequently, we have derived the full-length sequences of these and other ESTs and discovered that their putative gene products show significant sequence similarity to SCR and to each other throughout their carboxyl (C)-termini. This highly conserved region does not show significant similarity to members of any recognized gene family, indicating that these sequences define a novel gene family whose members we have called *SCARECROW-LIKE* (*SCL*). Recently, the importance of this family has been confirmed through the molecular analysis of two components of the gibberellin (GA) signal transduction pathway. The gene products of the *GIBBERELLIN-ACID INSENSITIVE* (*GAI*) and the *REPRESSOR of GA1* (*RGA*) loci, have been shown to be members of this family (Peng *et al.*, 1997; Silverstone *et al.*, 1998). For the family as a whole, we will use the acronym GRAS, based on the locus designations of these three genes (*GAI, RGA, SCR*).

At present the GRAS family includes 19 members in Arabidopsis. Here, we report the deduced amino acid sequences of the *SCL* gene products that we have sequenced, in addition to the expression of these sequences in Arabidopsis. Intriguingly, the majority of the *SCL* genes are expressed predominantly in the root, and

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one of these (*SCL3*) has a tissue-specific expression pattern in the root that is similar to that of *SCR*. The fact that the *SCR*, *GAI* and *RGA* gene products have diverse roles in fundamental processes in plant biology (*SCR* in pattern formation and *GAI*/*RGA* in signal transduction) suggests that other members of this family may also play important roles in the physiology and development of higher plants.

Results and Discussion

Identification of the SCLs

Three Arabidopsis ESTs whose predicted gene products bear striking similarity to SCR in a region termed the VHIID domain (Di Laurenzio *et al.*, 1996) were sequenced in their entirety. Comparisons of these sequences (now designated SCL1, SCL3 and SCL5) with SCR indicated that the similarity among the predicted gene products extended beyond the VHIID domain, in both the N- and C-terminal directions. Additional Arabidopsis ESTs were identified on the basis of their similarity to these highly conserved sequences (Table 1), and several were sequenced in their entirety (*SCL6*, *SCL7*, *SCL8*, *SCL9*, *SCL11*, *SCL13* and *SCL14*).

Database searches have also identified eight genomic sequences that potentially belong to this family (Table 1). Six of these are represented in the EST database, of which three (*SCL6*, *SCL9* and *SCL13*) correspond to ESTs that we have sequenced. Another three (*SCL4*, *SCL15* and *SCL19*) are represented by ESTs that were not initially identified as members based on the partial sequence available. Two of the genomic sequences (*SCL16* and *SCL18*) are not represented by ESTs and must be considered tentative members of the family. Unlike *SCR* (which contains a single intron), the genomic sequences for *SCL4*, *SCL6*, *SCL9* and *SCL15* all appear to contain a single open-reading frame encompassing all of the motifs present within the GRAS family.

The sequences for SCR, GAI and RGA have been reported previously (Di Laurenzio *et al.*, 1996; Peng *et al.*, 1997; Silverstone *et al.*, 1998). The deduced amino acid sequences for the *SCL13* and *SCL15* gene products have also been previously reported, as VHIID HOMOLOGOUS SEQUENCE4 (VHS4) and 5 (VHS5), respectively (Silverstone *et al.*, 1998). One additional member of this family has been deposited in the GenEMBL database and designated RGAL.

Definition of the GRAS family

The GRAS gene products share significant similarity throughout their C-termini, beginning at approximately 110 amino acid residues N-terminal of the highly conserved VHIID sequence and continuing throughout the C-terminal portion of the predicted products (Figure 1). This extensive sequence similarity can be subdivided into five distinct sequence motifs, found in the following order: leucine heptad repeat I (LHR I); the VHIID motif; leucine heptad repeat II (LHR II); the PFYRE motif; and the SAW motif (Figure 1).

The Leucine Heptad Repeats. The leucine heptad repeats (LHR I and LHR II) are unusual in structure. LHR I appears to consist of two repeat units (A and B in Figure 1b) that are separated by a spacer that often contains a proline residue, known to disrupt alpha-helical structures. The two units within LHR I are not in phase with each other. LHR IA is similar to LHRs found in other proteins, consisting of between three to five regular heptads. LHR IB is shorter, usually consisting of only two such repeats. LHR II is also unusual: although specific leucine heptad repeats can be identified in this region in nearly all members of the family, the number of repeats is small, usually two or three. The presence of leucine heptad repeats in the GRAS proteins suggests that these gene products may function as multimers (Hurst, 1994). The presence of four possible (albeit unusual) LHRs in some of the members suggests a potentially complicated higher order of interaction.

The VHIID Motif. The VHIID sequence is readily recognizable in all members of the family, although it is not absolutely conserved: substitutions of valine, isoleucine and leucine at the 1, 3 and 4 positions yield a number of permutations. Within the larger region that we term the VHIID motif, the P-N-H-D-Q-L residues are absolutely conserved (Figure 1). The spacing between the proline and asparagine residues is identical among all members, as is the spacing between the histidine, aspartate, glutamine and leucine residues. The VHIID motif is bounded at its C-terminus by a conserved sequence referred to as LRITG for simplicity (Figure 1). Most of the deviations from this consensus sequence represent conservative changes.

The PFYRE Motif. The PFYRE motif is not as well conserved at the sequence level as are the VHIID and SAW motifs (only the P is absolutely conserved) (Figure 1). Within the PFYRE domain, however, the sequences are largely co-linear and portions of this region show a high degree of sequence similarity among all members of the family.

The SAW Motif. The SAW motif is characterized by three pairs of absolutely conserved residues: R-E, W-G and W-W (Figure 1). The W-W pair found nearly at the C-terminus of these sequences shows absolute conservation of spacing, as does the W-G pair. The spacing between the W-G and W-W pairs, however, is not conserved.

Those GRAS gene products for which N-terminal sequence data exists beyond that shown in Figure 1 do

Table 1 Accession numbers and map positions of the GRAS sequences in Arabidopsis

Designation	Accession numbers	Map position
SCL1	E: Z25645/33772, B10318, B11686 Com: AF0360300	1: m235-g3829 (RI: JD1110)
GAI	Z34183, Z34599, T22782, Y11337, Y15193, B62171	1: ve006-ve007 (CIC3G6, 4H9, and 11C3)
SCL3	E: Z37192/Z37191, N96166, B20233, B18969 Com: AF0360301	1: m213 (CIC 1G8, 4H4, 8G4)
SCL4	E: Z46550, Z38048, Z38085, B22400, B23696 G: AB010700	5 (genomic clone)
SCL5	E: F13896/F13897, AA395075 Com: AF0360302	1: m213 (RI: JD4818)
SCL6	E: F13949 Com: AF0360303 G:AC004708 (WASHU003)	4: mi51 (CIC 2C7, 5B11, 5C11, 10C8) (genomic clone)
SCL7	E: R29793 Com: AF0360304	3: CDs4, m457 (CIC 8E2, 8E1, 9D1)
SCL8	E: T21627, H76979, N96767, T43670, AA395639, B77404 Com: AF0360305	5: PAP003 (CIC 11F10)
SCL9	E: T76186, T44774 G:AC004684, B25776 Com: AF0360306	2: ve018-nga168 (CIC 10F12) (genomic clone)
RGA	E: T45793, T46205, N96653, Y11336, Y15194	2: ve012 (CIC7C11, 2F4, and 6G2)
SCL11	E: T76483, AA394557, AA605493 Com: AF0360307	NP
SCL13 (*VHS4*)	F15454, N37425, AA720344, R29917 Com: AF0360308 G: Z97343	4: g4539-mi112 (CIC 4D3, 6G4, 2B8, 5E12, 7G8, 12B9) (genomic clone)
SCL14	E: W43803, W43538, AA042397 Com: AF0360309	NP
SCL15 (*VHS5*)	E: N65163 G: Z99708	4 (genomic clone)
SCL16 *RGAL*	G: AB007645 G: AJ224957	5 (genomic clone)
SCL18	E: B10115, B30030 G:AC002328	1: mi209,nga280,nga128 (BAC F20N2) (genomic clone)
SCL19	E: Z26055, B62171, B62460	
SCR	U62798	3: ve042-ve022 (CIC 11G5, 9D7)

'E' indicates an EST or a BAC end sequence. 'Com' indicates a complete EST sequence. 'G' indicates a genomic sequence.

not contain significant similarity among their N-termini, except in the case of GAI/RGA/RGAL (Peng *et al.*, 1997; Silverstone *et al.*, 1998; Truong *et al.*, 1997). The SCLs for which N-terminal sequence is available (SCL4, SCL6, SCL8, SCL9, SCL14 and SCL15) do not show any significant similarity to each other, to SCR, or to GAI/RGA/RGAL in this region (data not shown). The one common feature is that most of them contain homopolymeric stretches of certain amino acid residues (S, T, P, Q, G, E and/or H).

In summary, the GRAS gene products are characterized by a variable N-terminal region and a highly conserved C-terminal region. Importantly, the order of these motifs within each protein is the same. While the functions of the VHIID, PFYRE and SAW motifs are currently unknown, the absolute conservation of the residues in the VHIID and SAW motifs indicates that these residues are required for the activity of the GRAS gene products.

Other motifs in the GRAS family sequences

A putative nuclear localization sequence (NLS) that conforms to the consensus for bipartite NLS has been reported for SCR, GAI and RGA (Di Laurenzio *et al.*, 1996; Peng *et al.*, 1997; Raikhel, 1992; Silverstone *et al.*, 1998). Similar sequences are found in SCL14, SCL15 and RGAL. SCL4, SCL6 and SCL8 contain putative NLS conforming to the

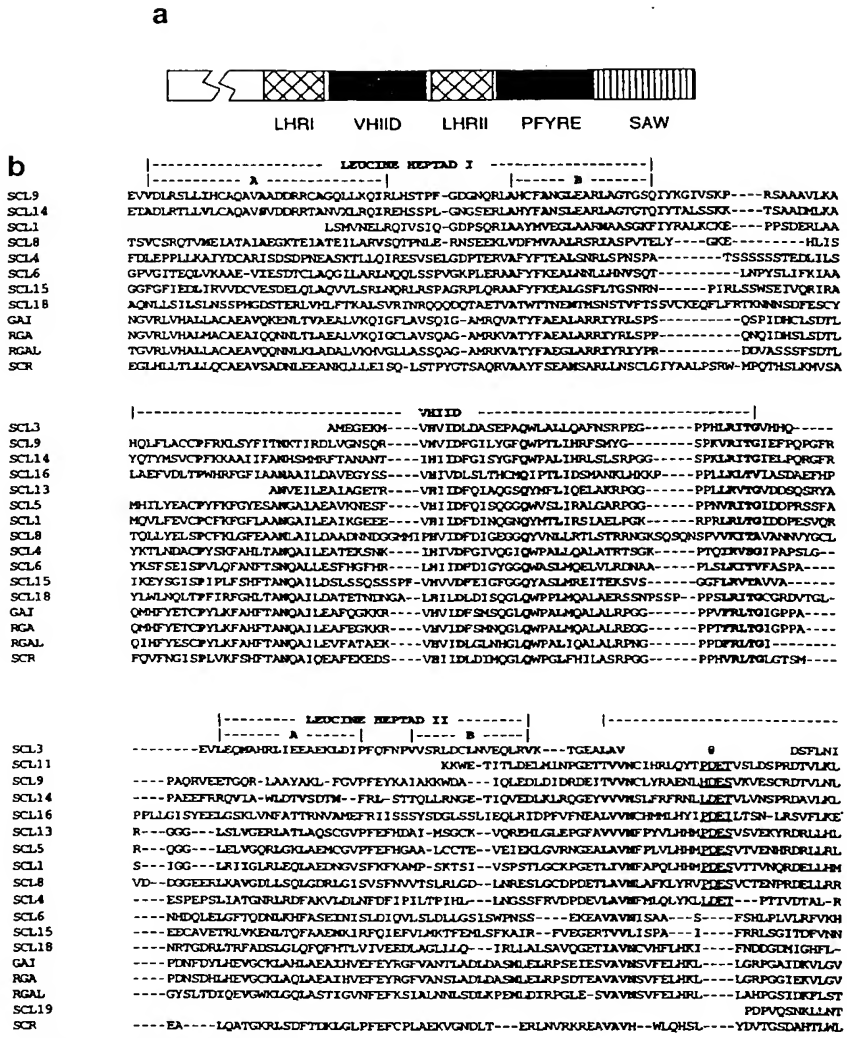

Figure 1. Alignment of the Arabidopsis GRAS gene products.
The highly conserved region of the GRAS products can be divided into five recognizable motifs, as indicated diagrammatically in (a). The deduced amino acid sequences for the SCLs are shown in (b), with the appropriate motif designations indicated above the sequence alignment. The absolutely conserved residues within the VHIID and SAW motifs are highlighted in bold, as are the hydrophobic residues of the leucine heptads, the P-F-Y-R-E residues of the PFYRE motif, and the two short sequences that define the end of the VHIID motif and the beginning of the PFYRE motif. The @ symbol in the alignment indicates the location of an apparent insertion in the *SCL3* gene. The deduced amino acid sequence of the insertion is shown at the bottom of the figure.

non-typical SV40-like NLS (Raikhel, 1992). Nuclear localization has been demonstrated indirectly for RGA, through use of an RGA-green fluorescence protein (GFP) fusion (Silverstone *et al.*, 1998).

The *GAI* and *RGA* gene products also contain a sequence that fits the consensus sequence (LXXLL) demonstrated to mediate the binding of steroid receptor co-activator complexes to nuclear receptors (Heery *et al.*, 1997; Peng *et al.*, 1997; Silverstone *et al.*, 1998; Torchia *et al.*, 1997). Sequences conforming to this consensus are also found in SCL4, SCL6, SCL15, RGAL and SCR. The significance of this sequence in plants is unknown.

The combination of motifs present in the GRAS family members suggests that they may act as transcriptional regulatory proteins. It is tempting to hypothesize further that the N-termini of the GRAS proteins function as activation domains: the variability of these sequences may result in the ability to mediate a number of different interactions with the basic transcriptional machinery and accessory proteins. The LHR I-VHIID-LHRII region may function as a DNA-binding domain, analogous to the bZIP protein–DNA interaction (Ellenberger *et al.*, 1992), with the LHRs mediating protein–protein interactions and the VHIID motif mediating protein–DNA interactions.

Figure 1b. Continued.

Evolutionary relationships

Comparison of conserved motifs among members of the GRAS family suggested that they could be grouped into distinct subsets. To determine the evolutionary relationship among these genes, the highly conserved sequences spanning the five motifs from the Arabidopsis members were analyzed by heuristic and bootstrap analyses to determine maximum parsimony. In the resulting phylogram, several distinct groups can be distinguished. These include: SCL11/14/9, SCL13/5/1, SCL4/7, SCL6/15 and GAI/RGA/RGAL/SCL19 (Figure 2). Three members, SCR, SCL3 and SCL8, do not group with any of the other sequences. The trees derived from analyses of amino acid and nucleotide sequences were nearly identical. Distance-based analyses yielded similar results.

Database searches identified putative GRAS family sequences in other plant species (rice, oat, alfalfa, maize, watermelon and *Brassica napus*). Recently, the *Lateral suppressor* (*Ls*) gene in tomato has been shown to be a new member of the GRAS family (Schumacher *et al.*, 1999). However, to date no significant similarity to the GRAS gene family has appeared among the sequences in any non-plant genome, including the fully sequenced yeast genome. Thus the GRAS gene family, like the AP2 family (Okamuro *et al.*, 1997; Weigel, 1995), appears to be restricted to higher plants.

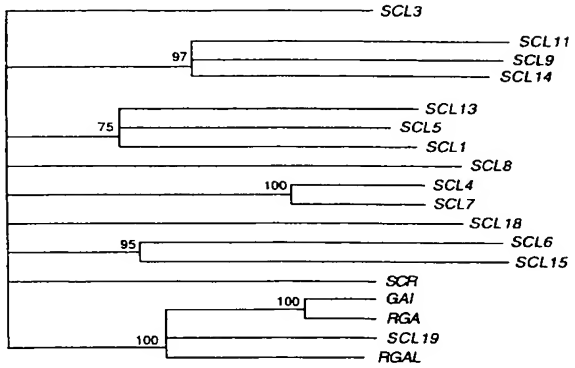

Figure 2. Phylogram of the Arabidopsis GRAS sequences.
The Arabidopsis GRAS nucleotide and deduced amino acid sequences were analyzed using the PAUP program. The results were similar for both parsimony and distance analyses. The tree is unrooted because there are no outgroup GRAS sequences available. The bootstrap values calculated from the nucleotide alignment are shown (maximum parsimony, replicates = 1000, gaps treated as fifth base). *SCL16* was not included in the analyses because of ambiguities in the available sequence.

RNA gel blot analyses

To begin to characterize the expression patterns of the *SCL* genes, RNA gel blot analyses of mRNA extracted from

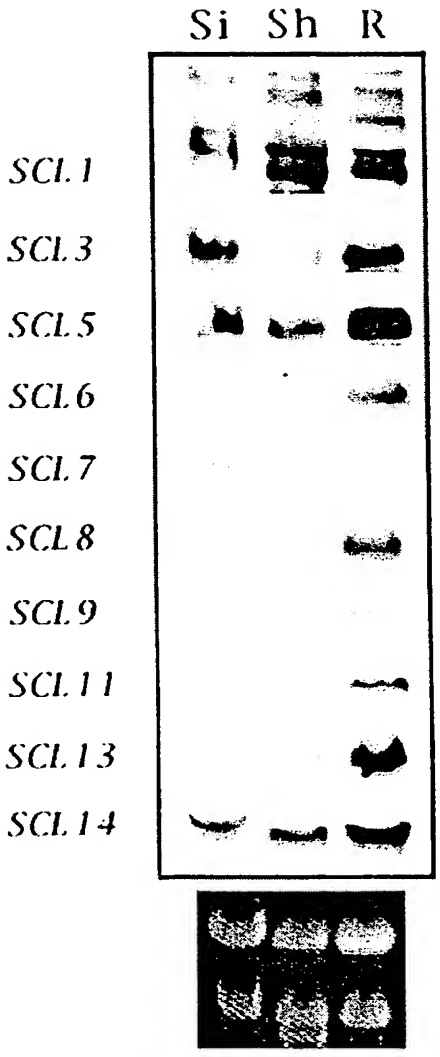

Figure 3. RNA gel blot analyses.
Total RNA from siliques (Si) and 14-day-old shoots (Sh) and roots (R) was isolated and analyzed by RNA gel blot hybridization with specific antisense digoxygenin-labeled probes. The *SCLs* analyzed are all expressed within the roots, and many of them are expressed in all of the organs tested. As the amount of mRNA loaded on the gels and the exposure times for all of these blots varied, direct comparisons of the levels of expression are not possible. Detection of *SCL1*, however, required significantly shorter exposures than the others, and *SCL6*, *SCL7* and *SCL9* required significantly longer exposures and more mRNA. A representative ethidium bromide-stained RNA gel is shown below as a loading control. Estimated sizes of the mRNAs for the *SCLs* are as follows: *SCL1*, 1.5/1.7 kb; *SCL3*, 1.8 kb; *SCL5*, 2.0 kb; *SCL6*, 2.4 kb; *SCL7*, 2.3 kb; *SCL8*, 2.7 kb; *SCL9*, 3.1 kb; *SCL11*, 2.1 kb; *SCL13*, 2.4 kb; and *SCL14*, 3.2 kb.

siliques, shoots and roots were performed. As can be seen in Figure 3, all of the *SCLs* analyzed are expressed in the root. *SCL6* and *SCL9* appear to be root-specific. A majority of the others show the highest level of expression in the roots (*SCL1* and *SCL7* are the exceptions). Although the levels of the *SCL* transcripts cannot be compared directly on these films due to variable exposure times and (in some cases) amounts of mRNA loaded on the gels, most of the *SCLs* appear to have similar levels of expression. The notable exceptions to this are *SCL1*, *SCL6*, *SCL7* and *SCL9*. Hybridization with the *SCL1* probe reproducibly resulted in multiple bands (Figure 3). Moreover, the exposure time required for detection with the *SCL1* probe was significantly shorter than that required for all of the other probes (minutes as opposed to hours). In contrast, detection of the *SCL6*, *SCL7* and *SCL9* transcripts required increased amounts of mRNA and longer exposure times, indicating significantly reduced levels of expression of these *SCLs* relative to the others. The *SCL* probes do not show any detectable cross-hybridization under the conditions used. This was initially indicated by the fact that Southern analyses using the *SCL1*, *SCL3* and *SCL5* probes resulted in a single hybridizing band in recombinant inbred analysis. Additionally, the unique messages detected using the *SCLs* as probes (with the exception of *SCL1*) vary in size: from 1.8 kb for *SCL3* to over 3.0 kb for both *SCL9* and *SCL14*. In summary, our expression analysis shows that many of the *SCL* sequences are expressed predominantly in the root, suggesting that a subset of these sequences may play important roles in root biology.

In situ *analyses*

RNA *in situ* hybridization with probes for *SCL3* were performed in order to begin to establish tissue-specific expression patterns. *SCL3* is expressed predominantly in the root endodermis, in a pattern strikingly similar to that of *SCR* (Figure 4). This pattern does not result from cross-hybridization with *SCR* or with other *SCLs*. This conclusion is supported by several facts. Most importantly, *SCL3* does not have significant stretches of absolute sequence homology at the nucleotide level with *SCR* or with any other member of the family. In addition, *SCL3* probes routinely result in only single hybridization patterns on both Southern and Northern blots. Finally, as noted above, the sizes of the transcripts hybridizing to different *SCL* probes can be clearly distinguished. The fact that *SCL3* is expressed in the root in a pattern very similar to *SCR* suggests that there exists a subset of *SCLs* involved in radial patterning and that *SCL3* plays a role in endodermal specification, perhaps by regulating the expression of *SCR* or by being regulated by *SCR*.

Significance of the GRAS gene products

In spite of many unknowns, the significance of the GRAS family is beginning to be understood. The *SCARECROW* gene, the defining member of this family, is absolutely required for the proper radial patterning of the root and

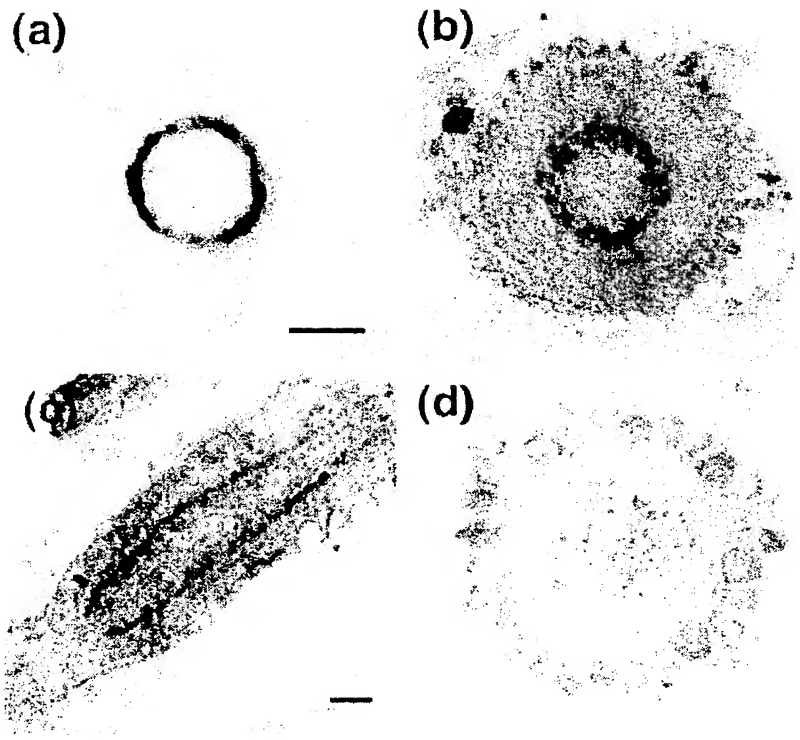

Figure 4. *In situ* hybridizations with *SCR* and *SCL3*. Transverse sections (a, b and d) and a longitudinal section (c) of 7-day-old roots were hybridized with either an antisense *SCR* riboprobe (a), an antisense *SCL3* riboprobe (b and c) or a sense *SCL3* riboprobe (d). Strong signal is observed in the endodermis with the antisense *SCR* probe and the antisense *SCL3* probe, but not with the sense *SCL3* probe. Scale bars in (a) and (c) are both 25 μm. The magnification is the same in panels (a), (b) and (d).

shoot in Arabidopsis (Fukaki *et al.*, 1998; Scheres *et al.*, 1995).

Plants mutant at the *GAI* locus are reduced in stature and do not respond to applications of exogenous GA, indicating that the GAI protein is involved in GA perception and response (Koornneef *et al.*, 1985). GAI may act as a negative regulator of cell elongation. It has been hypothesized that in wild-type plants, GAI represses cell elongation in the absence of GA (Peng *et al.*, 1997). The phenotype of the *rga* mutants indicates that RGA also negatively regulates GA perception and response (Silverstone *et al.*, 1997). The N-termini of GAI and RGA are highly similar, indicating that they may act through similar mechanisms. Deletion of five amino acids (DELLA) in the N-terminus in GAI results in the dominant phenotype, implicating this region in GA perception and response (Peng *et al.*, 1997). Therefore, these three prototypical GRAS gene products (*SCR*, *GAI* and *RGA*) establish that the members of the GRAS family play important roles in plant biology.

Experimental procedures

DNA sequencing, alignments and phylogeny

The *SCL* ESTs were obtained from the Arabidopsis Stock Center (Columbus, OH, USA) with the exception of *SCL6*, which was kindly provided by Thierry Desprez (INRA, France). The plasmid DNA was prepared by alkaline lysis (Sambrook *et al.*, 1989) and sequenced using Sequenase 2.0 (United States Biochemicals), according to the manufacturer's instructions. Sequences were translated using GeneWorks 2.0 (Oxford, UK) and aligned manually, based on alignments performed using GeneWorks and additional BLAST (Altschul *et al.*, 1997) searches. The sequences of the highly conserved region shown in Figure 1 were analyzed using the PAUP program. Trees were obtained using both maximum parsimony (gaps informative) and minimum evolution (distance) of both the protein and nucleotide sequences. Bootstrap analyses confirmed that the branches were strongly supported (all clades occurred at a frequency greater than 0.75, with 1000 replicates).

Mapping

The map positions of most of the sequenced Arabidopsis ESTs were determined using either the recombinant inbred lines (*SCL1*, *SCL5*) or PCR-based yeast artificial chromosome (YAC) library screening (*SCL3*, *SCL6*, *SCL7*, *SCL8*, *SCL9*, *SCL13*). Recombinant inbred mapping was performed as described previously (Di Laurenzio *et al.*, 1996). For YAC library screening primer pairs specific for each of the *SCLs* (18–21mers) were obtained from Ransom Hill. These primer pairs were utilized in a polymerase chain reaction with DNA from the CIC YAC library (Creusot *et al.*, 1995) using protocols and conditions described by Camilleri *et al.* (1998). The map positions of seven of the *SCL* genomic clones (*SCL4*, *SCL6*, *SCL9*, *SCL13*, *SCL15*, *SCL16*, *SCL18*) are known as a result of the Arabidopsis genome sequencing projects (Bevan *et al.*, 1998; Camilleri *et al.*, 1998; Schmidt *et al.*, 1995; Schmidt *et al.*,

1997; Zachgo *et al.*, 1996). *SCL11* and *SCL14* could not be placed on a YAC by the PCR-based method and the map positions of *SCL19* and *RGAL* are not known. The results of the mapping are summarized in Table 1.

RNA extraction and blot analysis

Total RNA was extracted from the roots and shoots of 14 day seedlings grown under standard sterile conditions (see Di Laurenzio *et al.*, 1996) and from siliques from plants grown on soil. Ten (*SCL1, SCL3, SCL5, SCL8, SCL11* and *SCL13*) or 18 (*SCL6, SCL7, SCL9, SCL14*) micrograms of total RNA were separated on formaldehyde gels, as in Di Laurenzio *et al.*, 1996. The RNA was transferred to HyBond-N (Amersham) and hybridized with digoxygenin-labeled single-stranded DNA probes using the GENIUS non-radioactive detection system (Bohreinger Mannheim), as per the manufacturer's instructions.

In situ *analyses*

Four- to seven-day-old light-grown seedlings grown under standard sterile conditions were fixed in paraformaldehyde, embedded in Paraplast Plus (Fisher), sectioned, and hybridized as reported in Di Laurenzio *et al.* (1996). Probes were digoxygenin-labeled using the protocol also described in Di Laurenzio *et al.* (1996).

Accession numbers for GRAS sequences in other plants

The GenBank/EMBL database accession numbers for the two members of this family that we sequenced from rice (OsSCL1) and maize (ZmSCL1) are AF067400 and AF067401, respectively. The following are accession numbers for ESTs that encode products with a VHIID motif: AA231684 (oat), AA751595 (rice), C72495 (rice), AA754049 (rice); with a SAW motif: AA231910 (oat), H74669 (Brassica), C28500 (rice); with similarities to the PFYRE motif: C20324 (rice), D15490 (rice), C28384 (rice). Additional ESTs encoding products with significant similarity to the GRAS sequences are C71780 (rice), AA660090 (watermelon), AA750594 (rice), and AA751136 (rice). The accession numbers of the ESTs that encode products with the DELLA sequence (like GAI and RGA) are AA660952 (watermelon) and D39460 (rice).

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Plant Biology

The *Lateral suppressor (Ls)* gene of tomato encodes a new member of the VHIID protein family

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ABSTRACT **The ability of the shoot apical meristem to multiply and distribute its meristematic potential through the formation of axillary meristems is essential for the diversity of forms and growth habits of higher plants. In the *lateral suppressor* mutant of tomato the initiation of axillary meristems is prevented, thus offering the unique opportunity to study the molecular mechanisms underlying this important function of the shoot apical meristem. We report here the isolation of the *Lateral suppressor* gene by positional cloning and show that the mutant phenotype is caused by a complete loss of function of a new member of the VHIID family of plant regulatory proteins.**

The pattern of shoot branching and the growth characteristics of lateral shoots determine to a large extent the growth habit of plants. In seed plants, shoot branching is initiated at the shoot apex with the formation of axillary meristems. In the axils of developing leaf primordia, distinct groups of meristematic cells, which are in direct continuity with the shoot apical meristem, can be identified by histological means, because of their dense cytoplasm and the low degree of vacuolation (1). This cell group proliferates and forms a dome-shaped axillary meristem, whose structure is very similar to that of the apical meristem of the primary shoot. After the formation of the first leaf primordia, development of these lateral buds often pauses due to the inhibitory effect of the shoot apex of the main shoot (2). In some plant species, the apical meristem of the primary shoot remains active throughout the life of the plant and continues to initiate the formation of lateral organs. In other plant species, the primary apical meristem at some point of development undergoes the transition to floral development or it aborts. In these cases, development is continued by one or few axillary meristems forming a sympodial shoot (e.g., *Lycopersicon esculentum* and *Petunia hybrida*).

Little is known about the genetic control of shoot branching. Mutants that exhibit either a reduced or an enhanced outgrowth of axillary buds have been described in various plant species (3). In other cases, the initiation of axillary meristems is blocked in some or most of the leaf axils. The *lateral suppressor (ls)* mutant of tomato is characterized by phenotypic abnormalities at different stages of development. During vegetative development the cells in the axils of leaf primordia fail to retain their meristematic character leading to the absence of side-shoots (4). However, at the transition to reproductive development, axillary meristems are initiated in the two leaf axils preceding the inflorescence. Whereas the uppermost axillary meristem develops into a sympodial shoot continuing the main axis of the plant, the second axillary meristem will develop into a side-shoot (4). Inflorescence development of homozygous *ls* plants is characterized by a lower number of flowers per inflorescence, the absence of petals (5), and a reduction in male and female fertility (6). The morphological defects of the *ls* mutant are accompanied by drastic changes in the levels of several plant hormones. In comparison to wild type, apices of homozygous *ls* plants contain much higher levels of auxins and gibberellic acid, whereas the levels of cytokinins are reduced (7). By studying the molecular processes underlying this complex phenotype, we hope to gain insight into important aspects of plant development. Based on previously reported genetic and physical mapping (8), we have isolated the *ls* gene by positional cloning. Sequence analysis of the mutant alleles reveals that the *ls* phenotype is caused by a complete loss of function of a member of the newly emerging family of VHIID regulatory proteins.

MATERIALS AND METHODS

Plant Materials. Tomato seed material of *L. esculentum* cv. Antimold B, Antimold B-*ls*[1] and Moneymaker was obtained from the Tomato Genetics Stock Center, Davis, CA. Tomato seeds of *L. esculentum* cv. Primabel and Primabel-*ls*[2] were obtained from J. Philouze (Institut National de la Recherche Agronomique, Montfavet, France). Plants were grown under standard glasshouse conditions with additional artificial light (16-h photoperiod) during the winter period.

DNA Isolation and Southern Blot Analysis. Plant DNA for PCR and Southern blot analysis was prepared as described (9). For Southern blot analysis, approximately 5 μg of genomic DNA was subjected to electrophoresis through 0.8% agarose, blotted to HybondN$^+$ membranes (Amersham Buchler, Braunschweig, Germany) and hybridized with radiolabeled probes. All standard techniques were carried out according to Sambrook *et al.* (10), unless otherwise stated.

RNA Isolation and Reverse Transcription (RT)-PCR Analysis. Total RNA was isolated by using the RNeasy system (Qiagen, Hilden, Germany) following the manufacturer's instructions. For RT-PCR analysis, 1 μg of total RNA was digested with DNaseI and reverse-transcribed by using the Superscript II polymerase (Life Technologies, Eggenstein, Germany) according to the manufacturer's instructions. The product of the first-strand cDNA synthesis reaction was amplified by PCR using the *Ls*-specific primers CD61–6 (5′-GGTGGCAATGTAGCTTCCAG-3′) and CD61–23 (5′-CCAGCTATTCAAATACGCCAG-3′). Amplification of ac-

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Abbreviations: *ls*, *lateral suppressor;* RT, reverse transcription; RACE, rapid amplification of cDNA ends; YAC, yeast artificial chromosome; SCR, Scarecrow; GAI, Gibberellin insensitive; RGA, repressor of ga1-3.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF098674).

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tin cDNA by using primers specific for the potato gene PoAc101 (11) was performed as a control to ensure that equal amounts of cDNA were added to each PCR.

cDNA Isolation and Rapid Amplification of cDNA Ends (RACE) Experiments. A tomato (cv. VFNT Cherry) shoot tip cDNA library (12) was screened by using either yeast artificial chromosome (YAC) 61–5, the whole cosmid contig (Fig. 1*A*) or the insert of cosmid G as a probe. In each experiment at least 1×10^6 plaques were screened.

For RACE experiments (13), first-strand cDNA synthesis was performed as described above for RT-PCR analysis. RACE experiments were performed by using the RACE system of Life Technologies according to the manufacturer's instructions. For 5′ RACE, the standard adapter primer BRL-UAP and the *Ls*-specific primers CD61–30 (5′-TGATGGACTAACCGTTCAG-3′), CD61–11 (5′-AGCTAATGAGTAGCTGGCGG-3′), and CD61–31 (5′-TTGGAGTTGTTTCAACAGG-3′) were used. Amplified fragments were cloned into the pGEM-T (Promega) plasmid vector.

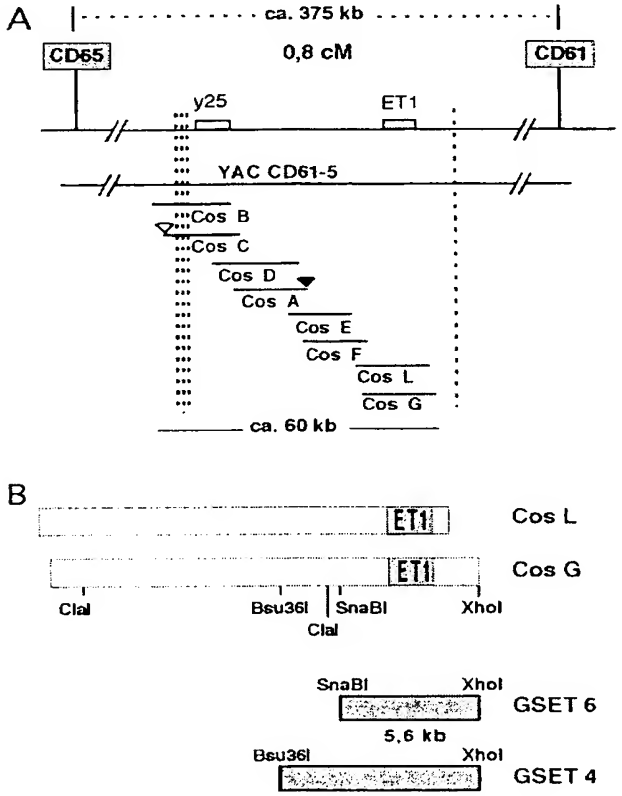

FIG. 1. Physical map of the *Ls* region. (*A*) Schematic representation of the cosmid contig around the *Ls* locus. The YAC and cosmid clones are shown as horizontal lines. The dashed vertical lines indicate the approximate positions of recombination breakpoints. Open boxes represent cDNAs, and flanking restriction fragment length polymorphism markers are displayed as shaded boxes. Cosmid end probes used for orientation of the cosmid contig are indicated as an open (C-for) or closed triangle (A-rev). (*B*) DNA fragments used for complementation experiments. The open bars represent the cosmid clones G and L. Shaded bars indicate different DNA fragments of cosmid G used for complementation. The position of the ET1 cDNA is displayed as a shaded box.

Isolation of YAC Clones. The PCR primers CD61-F, CD61-R, CD65-F, and CD65-R deduced from the restriction fragment length polymorphism markers CD61 and CD65, respectively (8), were used to screen a pooled tomato YAC library (14). To identify a YAC clone spanning the CD61-CD65 interval (8), CD61-positive clones also were analyzed with CD65 primers, and vice versa. After separation of agarose-embedded yeast chromosomes by pulsed field gel electrophoresis, YAC DNA was extracted by Gelase (Biozym, Hess. Oldendorf, Germany) digestion, radioactively labeled by using standard procedures, and used to screen a cDNA library.

Construction of a Binary Vector Cosmid Library. Genomic DNA of *L. esculentum* cv. Moneymaker was partially digested with *Mbo*I, and a 17- to 23-kb size fraction was ligated into the *Bam*HI site of the binary cosmid vector pCLD04541 (15), packaged by using commercial extracts (Gigapack II, Stratagene), and transfected into the *Escherichia coli* strain SURE (Stratagene). Approximately 250,000 recombinant clones with an average insert size of 20 kb representing more than five genome equivalents were divided into 100 pools. Screening of the library was done by PCR analysis of DNA from each pool followed by conventional colony filter hybridization.

Tomato Transformation. DNA fragments were cloned into the binary vectors pCLD04541 (15) or pGPTV-Kan (16) and transferred into the *Agrobacterium tumefaciens* strains GV3101 (17) or LBA4404 (18). Transformation of tomato leaf explants was done as described (19).

DNA Sequencing and Analysis. DNA sequencing was done by using the PRISM Ready Reaction Terminator Cycle Sequencing system (Applied Biosystems). Reactions were run on an Applied Biosystems 373A or 377XL DNA sequencer. Computer analysis was performed by using the following software: WISCONSIN package, Version 9.1, Genetics Computer Group, Madison, WI.

RESULTS

Establishment of a Cosmid Contig from the *ls* Region. Previous work had mapped the *Ls* locus to an interval with a maximum size of 375 kb at the top of chromosome 7 defined by the restriction fragment length polymorphism markers CD61 and CD65 (8). Subsequently, CD61-specific primers were used to screen a pooled tomato YAC library (14). Among five clones isolated, YAC CD61–5 was shown by Southern analysis to hybridize to both CD61 and CD65, and therefore its 320-kb insert should encompass the whole CD61-CD65 interval including the *Ls* locus.

DNA of YAC CD61–5 was isolated by pulsed field gel electrophoresis and used as a probe to screen a tomato shoot tip cDNA library (12). Among the isolated cDNA clones, representing at least 29 transcripts, only one, y25, showed cosegregation with the *Ls* locus and was therefore used as an anchor to establish a cosmid contig of the *Ls* region on chromosome 7. Screening of a genomic cosmid library with y25 resulted in an initial set of four overlapping cosmid clones. To orient this set of overlapping cosmid clones relative to the genetic map, end probes of the contig were mapped in a population of recombinants harboring recombination breakpoints around the Ls locus (8). Whereas probe C-for detected three recombinants in the interval between *Ls* and CD65, the end probe A-rev of cosmid A cosegregated with the *Ls* locus (Fig. 1*A*), indicating that we had to extend the contig in the direction of CD61. After two consecutive rounds of isolating overlapping cosmid clones, a contig covering ≈60 kb of genomic DNA was established (Fig. 1*A*).

Functional Complementation of the ls^1-Mutant. To test for complementation, cosmids (Fig. 1*A*) were introduced into leaf discs of the *lateral suppressor* mutant (ls^1/ls^1) by using the *Agrobacterium* strain LBA4404. Transgenic plants were screened for development of side-shoots and petals. A total of

50 transgenic plants harboring the cosmids A, B, C, D, E, and F or the vector plasmid pCLD04541 produced neither side-shoots nor petals (Table 1). However, eight of 16 independent transgenic plants transformed with either cosmid G or cosmid L did complement the mutant phenotype. Southern blot analysis revealed that only those plants showing complementation contained a complete T-DNA copy. Three transgenic plants harboring an intact copy of cosmid G developed side-shoots in almost every leaf axil and produced a whorl of petals on all flowers (Fig. 2). In contrast, we found that the five transgenic lines harboring a complete copy of cosmid L developed side-shoots in only a fraction of their leaf axils (69% in one transgenic line) and also showed an incomplete restoration of the flower phenotype. Because this finding may indicate that the *Ls* gene of cosmid L contains a mutation or that a regulatory element is missing, subsequent experiments were done by using cosmid G.

To define the position of the *Ls* gene more precisely, we tested subfragments of cosmid G for complementation of the ls^1 mutant. Whereas introduction of the 10.3-kb *Cla*I fragment (Fig. 1*B*) did not complement the ls^1 mutant, the subfragments GSET 4 (8.4 kb) and GSET 6 (5.6 kb) restored the wild-type phenotype (Table 1). This result demonstrated that the *Ls* gene is located within the 5.6-kb *Sna*BI–*Xho*I fragment of cosmid G.

Inheritance of the complementation phenotype was analyzed in the transgenic line 9620 harboring a single copy of cosmid G. Among 28 plants of the self-pollinated progeny of 9620, we found 20 plants showing complementation and eight plants with the *ls* phenotype. Resistance to kanamycin was observed only in those plants showing complementation. This result is consistent with the assumption that a single-copy T-DNA insertion, segregating in a Mendelian fashion, rescues the *ls* phenotype.

Identification and Characterization of the *Ls* gene. Subfragments covering almost the complete cosmid G were used as probes to screen a shoot tip cDNA library prepared from RNA of vegetative and floral shoot tips. Among 10^6 clones tested, we identified two cDNA clones of which only one, ET1, was found to be derived from the 5.6-kb *Sna*BI–*Xho*I fragment showing complementation of the ls^1 mutant. DNA sequence analysis revealed that ET1 contains an ORF starting with the first nucleotide of the cDNA and ending with a stop codon at position 1415 (GenBank accession no. AF098674), followed by an untranslated 3′ region of 271 bp, and a poly(A) tail.

To determine the 5′ end of the transcript, three independent products obtained in 5′ RACE experiments were sequenced. All three products started at the same base pair (position 1), suggesting that this position corresponds to the 5′ end of the transcript. The ATG initiating the ORF (position 131) is preceded by several stop codons in all three frames, strongly suggesting that this ATG corresponds to the translation start site. From these experiments, we conclude that the *Ls* transcript has a length of ≈1,7 kb and contains an ORF with a coding capacity for 428 aa. Comparison of the cDNA and the corresponding sequence of the genomic DNA revealed no sequence deviation between the cDNA and its genomic counterpart, which demonstrates that the *Ls* gene does not contain an intron.

Table 1. Complementation experiments

Construct	No. of transgenic plants	No. of plants showing complementation
pCLD04541	8	0
Cosmid A	5	0
Cosmid B	15	0
Cosmid C	5	0
Cosmid D	7	0
Cosmid E	2	0
Cosmid F	8	0
Cosmid G	5	3
Cosmid L	11	5
GSET4	2	2
GSET6	13	13
*Cla*I	5	0

To prove that the ORF identified corresponds to the *Ls* gene, we searched for sequence alterations in the mutant *ls* alleles. For this purpose, PCR products derived from genomic DNA of the ls^1 and ls^2 mutant were sequenced. A deletion of ≈1.5 kb was detected in ls^1, removing the first 185 aa of the predicted protein and 995 bp of the leader and the presumptive promoter region. In the ls^2 allele, the nucleotide sequence CAACAGCG (position 203–210) is replaced by TA-AAAACGGAA. The C to T transition at position 203, which changes a Q to a stop codon, is predicted to cause a premature termination of translation after 24 aa. The results of the complementation experiments together with the sequence analysis of the wild-type and the two mutant *ls* alleles demonstrate that we have isolated the *Ls* gene.

Ls Is a New Member of the VHIID Family. Comparison of the Ls protein sequence to the databases resulted in a list of proteins with considerable sequence similarity. Besides several sequences of unknown function identified in sequencing projects, this list includes three genes identified recently in *Arabidopsis thaliana*, which belong to the VHIID family of regulatory proteins: Scarecrow (SCR; ref. 20), Gibberellin insensitive (GAI; ref. 21), and repressor of gal-3 (RGA; ref. 22). With the exception of the N terminus (amino acids 1–47) similarity between Ls and the other members of this family extends over the whole length of the ORF (≈35% sequence identity by using the computer program FASTA). As we have recently isolated a clone from *A. thaliana* with considerably higher sequence similarity to *Ls* than either *SCR*, *GAI*, or *RGA* (unpublished results), we can exclude the possibility that *Ls* represents the tomato homolog of one of these genes.

The VHIID motif of unknown function, after which this family of proteins was named, is included in a modified version (IHIVD) in a region (amino acids 152–191 in Ls) showing the highest conservation between the different members of the family (Fig. 3). Only two additional sequence motives described for one or more of the three related proteins are conserved in Ls: the second leucine heptad repeat found in all four related genes and the LXXLL-motif (^{267}LHRLL^{271}) found in GAI and RGA that was recently shown to mediate interaction of transcriptional coactivators with nuclear receptors (23). All other sequence motives described for one or more of the related proteins, like the nuclear localization signal, the first leucine heptad repeat, or the bZIP-like domain, are not found in Ls, which makes a functional conservation unlikely.

The N terminus of the Ls protein is considerably shorter than the N termini of the three related proteins (47 aa in Ls, 288 aa in SCR, 166 aa in GAI, and 219 aa in RGA), and it does not show obvious sequence conservation, but it shares with the other sequences clusters of serine and threonine residues (amino acids 7–46). A second region with clustered serine and threonine residues is found in Ls between amino acids 106 and 128.

RT-PCR Detection of *Ls* mRNA. The identification of only one hybridizing cDNA clone from a shoot tip cDNA library among ≈10^6 plaques tested suggested that the steady-state levels of the *Ls* transcript are very low. This initial observation was corroborated by the finding that the *Ls* mRNA was not detectable in Northern blot hybridization experiments. Therefore, RT-PCR analysis was performed with total RNA from different plant organs. The *Ls* transcript was detected in shoot tips, flowers, roots, and young leaves but not in internodes (Fig. 4*A*). Because of the inherent characteristics of the RT-PCR technique, the observed

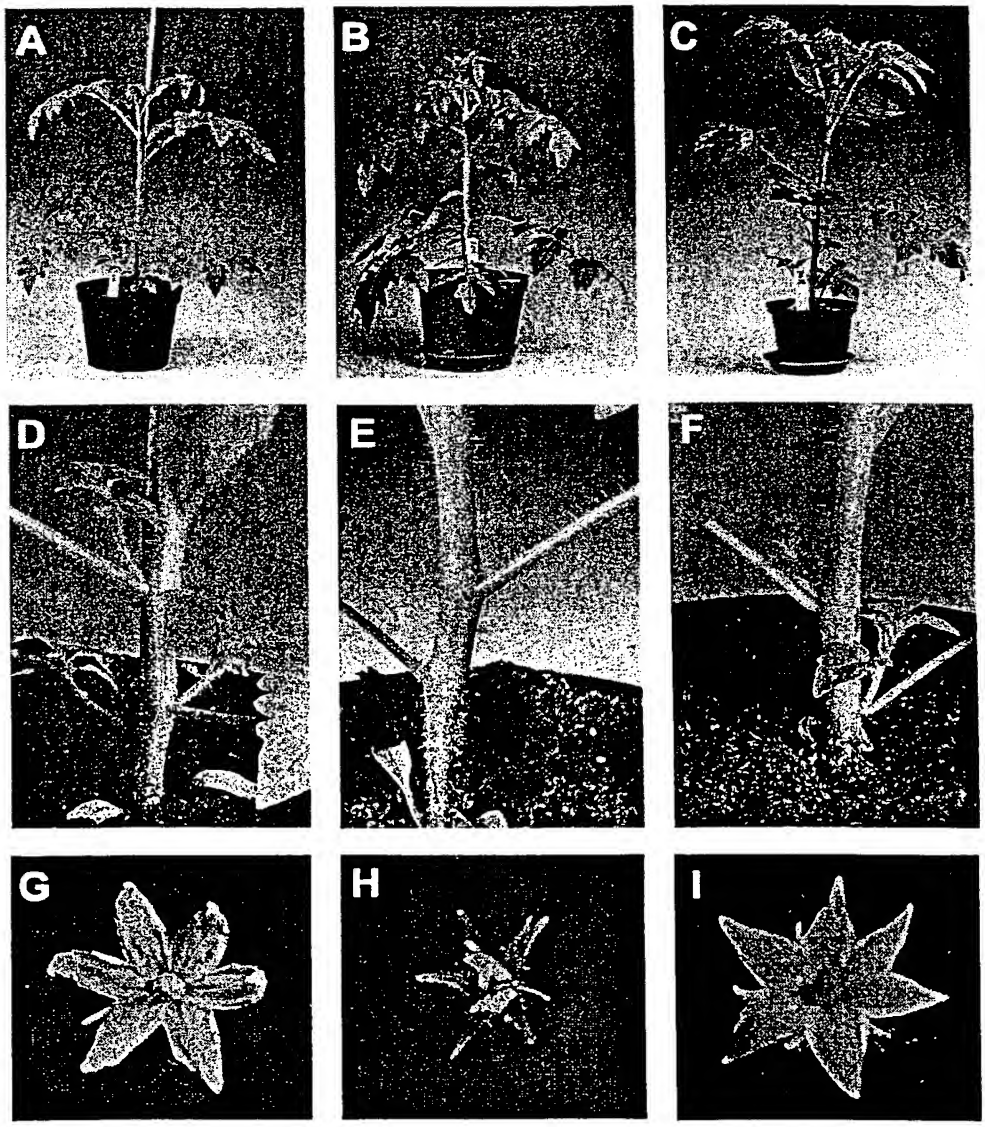

FIG. 2. Functional complementation of ls^1. Comparison of phenotypes of Antimold B (*A*, *D*, and *G*), Antimold B-ls^1 (*B*, *E*, and *H*), and Antimold B-ls^1 transformed with cosmid G (*C*, *F*, and *I*). The pictures show the growth habit (*A-C*), a close up of leaf axils (*D-F*), and a close-up of a flower (*G-I*).

quantitative differences in mRNA levels have to be treated with caution.

To test for the presence of a transcript from the two mutant alleles, total RNA extracted from young leaves of plants homozygous for either the wild-type *Ls* allele, ls^1, or ls^2, respectively, was analyzed by RT-PCR. In ls^1/ls^1 plants, no mRNA was detected whereas ls^2/ls^2 and wild-type plants contained transcripts of equal size (Fig. 4*B*). This result is in agreement with the finding that the ls^1 allele contains a deletion removing part of the ORF as well as the presumptive promoter region and demonstrates that ls^1 is clearly a null allele.

DISCUSSION

We have isolated the *Ls* locus by positional cloning and demonstrated the identity of the *Ls* gene by complementation and sequence analysis of the two existing mutant alleles. The identical phenotypic defects of the mutant alleles are in both cases because of a complete loss of function of the Ls protein caused by a deletion in ls^1 and the introduction of a stop codon after only 24 aa in ls^2.

The protein encoded by the *Ls* gene shares significant sequence similarity with members of the emerging family of plant VHIID proteins. In addition to the characteristic VHIID domain of unknown function, the previously described members of this family, SCR (20), GAI (21), and RGA (22), show a number of features pointing to a potential role as transcriptional regulators. In the well conserved C-terminal part only a leucine heptad repeat (amino acids 206–226) and an LXXLL (aa 267–271) motif are conserved between Ls and other family members. The LXXLL motif has been shown to be involved in binding of steroid receptor coactivators to the respective steroid receptors (23). However, the significance of this motif for plants is questionable because it occurs frequently and the presence of nuclear receptor-like proteins in plants has yet to

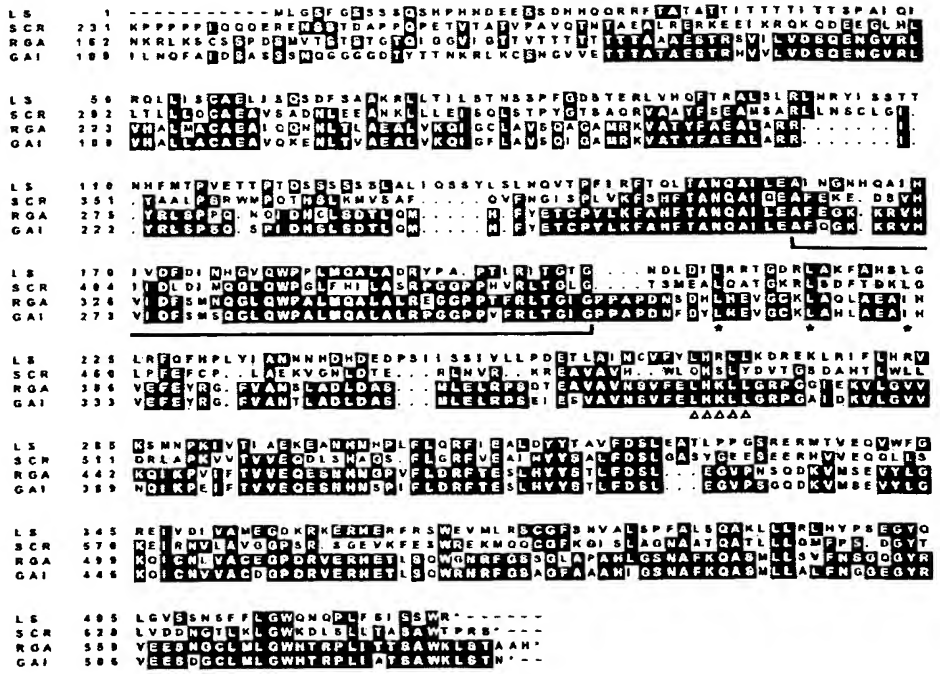

FIG. 3. Sequence analysis of the *Ls* gene. Alignment of the amino acid sequences of the Ls protein with the *Arabidopsis thaliana* proteins SCR (19), RGA (21), and GAI (20). Identical residues are displayed in reverse type and similar residues are in gray boxes. The VHIID domain is underlined, the leucine residues defining the conserved leucine heptad repeat are identified by asterisks, and the LXXLL motif by triangles.

be shown. Although the N-terminal domains of members of this family are of varying length and are not similar in sequence, the presence of short homopolymeric stretches of serine and threonine residues is a feature that the Ls protein shares with RGA and SCR and to a lesser extent with GAI. Such homopolymeric stretches have been found in the activation domains of transcription factors, and it has been demonstrated that serine- or threonine-rich stretches can serve as targets for the modification with *N*-acetylglucosamine residues leading to changes in activity of the respective proteins (24). Such modifications may be introduced by proteins like the SPINDLY protein of *A. thaliana*, which is involved in the regulation of GA signal transduction and shares sequence homology with *N*-acetylglucosamine transferases from animals (25). As the predicted Ls protein lacks a putative NLS signal, we do not have strong indications for a role as a transcriptional regulator. The presence of a conserved leucine heptad repeat however makes it conceivable that it interacts with related proteins, which themselves act as transcriptional regulators.

The fact that the protein encoded by the *Ls* gene is related to two proteins involved in negative regulation of GA signal transduction (GAI, RGA) lends support to a model that postulates a role for the Ls protein in a mechanism of localized regulation of GA responsiveness. This view is supported by the finding that the *ls* mutant is characterized by a severe imbalance of the major plant hormones (7). Among the hormones that show altered levels, GA is of particular interest as some aspects of the *ls* phenotype, like reduced seed germination and petal development, are known to be influenced by GA (26, 27). To maintain their undifferentiated state, meristematic cells must be protected from hormonal signals inducing differentiation in surrounding cells. One way to achieve this is through a localized negative regulation of the GA signal transduction pathway. The low abundance of the *Ls* mRNA has so far not allowed us to support this model by analyzing the expression on the *in situ* level, but RT-PCR analysis shows *Ls* expression

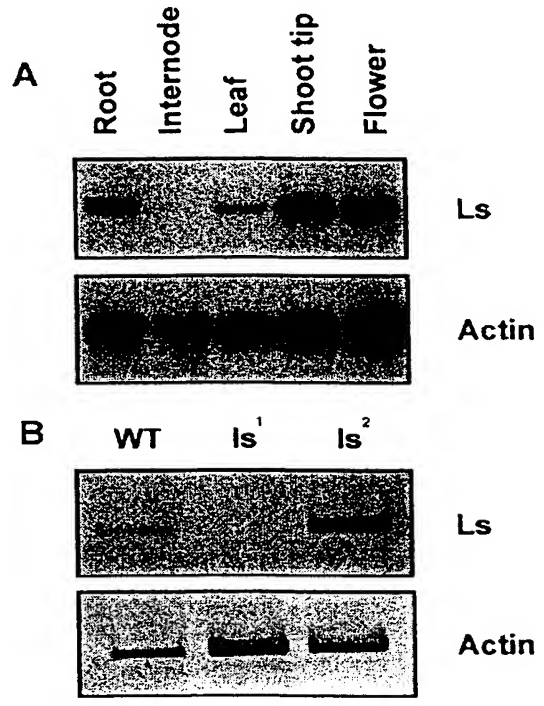

FIG. 4. RT-PCR detection of *Ls* mRNA in different plant organs. (*A*) Total RNA from different plant organs was analyzed by RT-PCR, and the PCR products were hybridized to the *Ls* cDNA as probe. Amplification of actin cDNA was used to ensure that equal amounts of cDNA were added to each PCR reaction. (*B*) Total RNA was extracted from leaves of plants homozygous for Antimold B-*Ls* (Wt), Antimold B-ls^1, and Primabel-ls^2 and then analyzed by RT-PCR.

in tissues that include the primordia affected in *ls* mutant plants. The dramatic increase in GA levels found in different organs of the *ls* mutant (7) could be ascribed to a perturbation of feedback inhibition of GA synthesis as it is assumed in the cases of GA-insensitive mutants, which contain elevated levels of GA (28, 29). The imbalances in the levels of the other major plant hormones may be either the result of an interrelation of the metabolisms of the different plant hormones or may indicate that the Ls protein is involved in different signal transduction pathways. The isolation of the *Ls* gene allows us to test our model and address the question of the causal relationship between morphological defects and hormonal imbalances on the molecular level.

Despite carrying null alleles, *ls* mutants are still able to form axillary meristems in the axils of the two leaf primordia preceding the inflorescence. This observation indicates that the Ls protein is not absolutely required for axillary meristem formation. It seems possible that, with the transition of the vegetative shoot apical meristem into an inflorescence meristem, the strength of the differentiation signal is reduced so that a protection of the cells forming the axillary meristem is no longer needed. Alternatively, it could be assumed that the mechanisms underlying the formation of the sympodial sideshoots are different and that the *Ls* gene is not involved in this process.

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EOD records of its three closest relatives[3] (Fig. 4b). The EOD of the monophasic *Brachyhypopomus* species is similar to that of the electric eel in waveform, duration and spectrum[24] (Fig. 4a). The resting discharge rate was low (8–13 Hz), resembling an alert electric eel in both rate and variability[24]. Calibrated daytime EOD amplitudes were 1.5–1.8 mV cm^{-1} at 10 cm, which is between five and ten times greater than specimens of its sister species[3] *Brachyhypopomus* sp. 2 (Fig. 4b). Thus the monophasic *Brachyhypopomus* species appears to have lost the second phase of its EOD and boosted EOD amplitude, consistent with Hagedorn's proposal that this fish is a batesian electric mimic of the sympatric electric eel. Further confirmation of the hypothesis could come only from experiments showing mutual avoidance of electric eels and monophasic *Brachyhypopomus* by an electroreceptive predator.

In summary, predation avoidance is the strongest candidate as the driving force for the initial evolution of EOD complexity, in particular, the transition from primitive monophasy to biphasy. This conclusion is supported by three lines of evidence: (1) spectral comparison of monophasic and biphasic EODs; (2) demonstration that biphasic pulses are less detectable by a known electroreceptive predator; and (3) examples of specific adaptations (high voltage, geographic isolation and mimicry) that protect species with monophasic EODs. Sexual dimorphism in the second EOD phase of *Brachyhypopomus* spp. (Fig. 2) seems to be the secondary modification of an adaptation for signal crypsis. Evolutionary escape from predation has been cited as a key factor promoting adaptive radiation[25]. Thus spectral shifting may have contributed to the success of this order in tropical South America. A key question is whether signal multiphasy evolved in any gymnotiforms outside the geographic range of their electroreceptive predators. Several extant multiphasic gymnotiform taxa extend beyond the range of large electroreceptive predators[23] (O. Macadar, personal communication), but their centres of distribution lie in the predator-rich continental tropics and none could be argued to represent an independent origin of multiphasy.

A parallel story may emerge from Africa, where mormyrid electric fish have undergone extensive radiation and an electroreceptive predator, the catfish *Clarias*, serves as their major predator[26,27]. Nor are electric fish entirely unique in having protective signal adaptations exploited by sexual selection. Ctenuchid moths evolved acoustic signals to alert predatory bats of their toxicity, and these signals have likewise been co-opted for mate attraction[28,29]. □

Methods

An electric eel 1 m long was trained to receive food (goldfish) when it approached any playback of an electric field in its round aquarium (120 cm diameter, 60 cm deep). We played electric stimuli from a DC-coupled 5-cm carbon dipole at calibrated intensities equivalent to natural EODs[4]. DC offset at 10 cm from the dipole centre was less than 0.05 μV cm^{-1}. Training stimuli included a wide variety of monophasic and biphasic digitized EODs. Experimental stimuli included the biphasic EOD of a female *Brachyhypopomus pinnicaudatus* and the same EOD with the second phase digitally removed (Fig. 3c, d). Trials were sequenced randomly. Playbacks of 1 min duration began while the eel rested on the tank bottom, at a distance of more than 60 cm from the electrode. A 'blind' assistant rewarded all electrotactic approaches with food. In the first set of trials, playback rate simulated a repeated social signal, 0.5 s at 50 Hz alternated with 0.5 s at 200 Hz. In a second set of trials playing the truncated stimulus only, rate modulation had no effect on frequency of approach (19/21 trials with rate modulation compared with 19/20 trials at 50 Hz). I measured EOD amplitudes (Fig. 4b) by methods published previously[4].

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'Green revolution' genes encode mutant gibberellin response modulators

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World wheat grain yields increased substantially in the 1960s and 1970s because farmers rapidly adopted the new varieties and cultivation methods of the so-called 'green revolution'[1–4]. The new varieties are shorter, increase grain yield at the expense of straw biomass, and are more resistant to damage by wind and

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rain[3,4]. These wheats are short because they respond abnormally to the plant growth hormone gibberellin. This reduced response to gibberellin is conferred by mutant dwarfing alleles at one of two *Reduced height-1* (*Rht-B1* and *Rht-D1*) loci[4,5]. Here we show that *Rht-B1*/*Rht-D1* and maize *dwarf-8* (*d8*)[6,7] are orthologues of the *Arabidopsis Gibberellin Insensitive* (*GAI*) gene[8,9]. These genes encode proteins that resemble nuclear transcription factors and contain an SH2-like[10] domain, indicating that phosphotyrosine may participate in gibberellin signalling. Six different orthologous dwarfing mutant alleles encode proteins that are altered in a conserved amino-terminal gibberellin signalling domain. Transgenic rice plants containing a mutant *GAI* allele give reduced responses to gibberellin and are dwarfed, indicating that mutant *GAI* orthologues could be used to increase yield in a wide range of crop species.

Gibberellin is an essential endogenous regulator of plant growth[11]. *Rht-B1b* and *Rht-D1b* are semidominant, altered function (rather than loss-of-function) mutant alleles of the *Rht-1* height-regulating genes of wheat. These mutant alleles reduce plant height (Fig. 1a), reduce responses to gibberellin and increase *in planta* gibberellin levels[4,5,12,13]. These properties are also characteristic of mutant alleles of maize *d8*[6,7,14] and of the *Arabidopsis gai* allele[8,9,15], indicating that these mutant alleles might define orthologous genes that are involved in gibberellin signalling. *GAI* (the wild-type allele) encodes a protein (GAI) containing features that are characteristic of transcription factors[9]. The *gai* allele encodes a mutant protein (gai), lacking 17 amino acids from near the amino terminus, that is thought to confer the altered gibberellin responses characteristic of the *gai* mutant[9]. Database searches revealed a rice expressed-sequence tag (EST; D39460) that encodes a potential polypeptide containing a sequence nearly identical to these 17 amino acids[16]. We used this EST to investigate whether the dominant dwarfing mutant alleles of *GAI*, *Rht-1* and *d8* identify orthologous genes in *Arabidopsis*, wheat and maize.

D39460 was used to isolate wheat complementary DNA C15-1. The genome of bread wheat is hexaploid, consisting of three homoeologous chromosome sets (the A, B and D genomes). Analysis of lines lacking particular chromosomes (nullisomic) showed that C15-1 hybridized to genomic DNA fragments derived from wheat chromosomes 4A, 4B and 4D (Fig. 1b), correlating with the location of the *Rht-1* alleles (all known mutant *Rht-1* alleles are on chromosomes 4B or 4D; ref. 5). Furthermore, restriction-

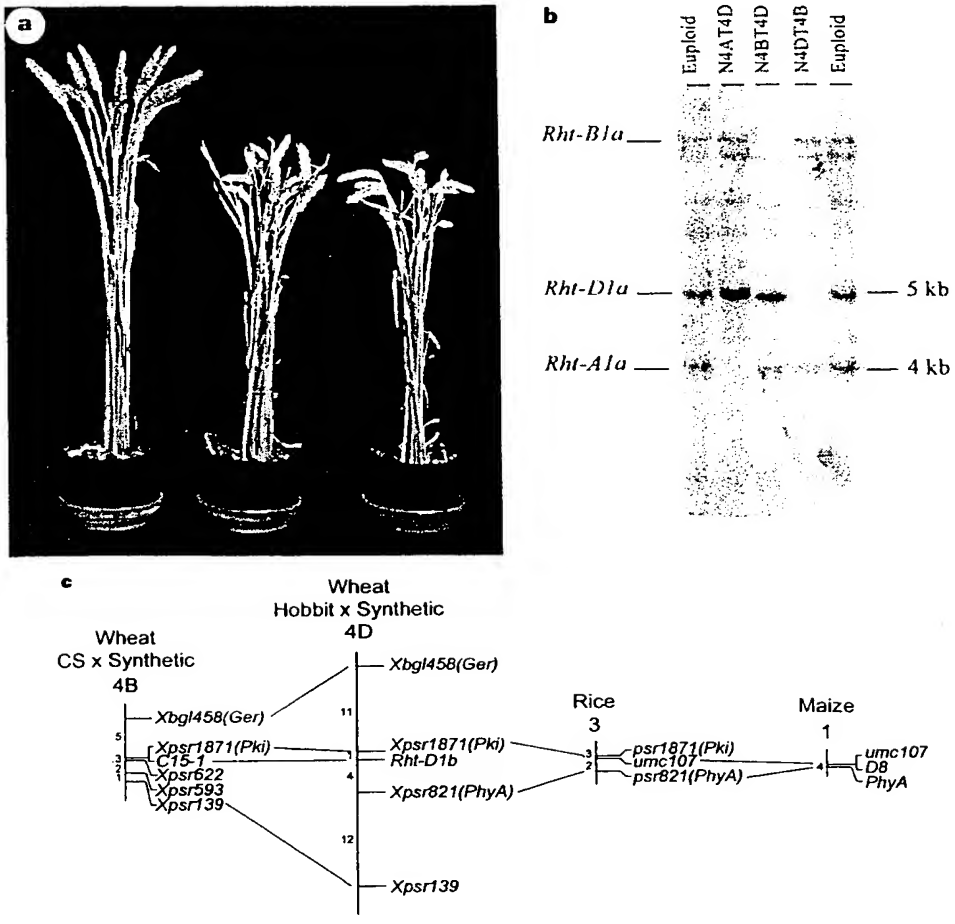

Figure 1 cDNA C15-1 maps to the *Rht-1* locus. **a**, Near-isogenic dwarf wheat lines: left, tall control (var. Mercia); centre, semi-dwarf *Rht-B1b*; right, semi-dwarf *Rht-D1b*. **b**, Gel-blot hybridization of C15-1 with *Dra1*-digested DNA from wheat lines lacking individual group 4 chromosomes (nullisomic 4A-tetrasomic 4D, N4AT4D; nullisomic 4B-tetrasomic 4D, N4BT4D; nullisomic 4D-tetrasomic 4B, N4DT4B), and euploid control (all var. Chinese Spring). Hybridizing fragments were assigned to chromosomes (4A, 4B and 4D) as shown. **c**, Partial linkage maps of wheat chromosomes 4B (ref. 26) and 4D, rice chromosome 3 (ref. 28), and maize chromosome 1 (ref. 29) showing the colinearity between regions containing C15-1, *Rht-D1b* and *D8-1*. A putative maize *d8* genomic fragment (see text) also displayed tight linkage with *umc107* (not shown). Wheat 4B data are from the F_2 of a Chinese Spring (CS) × Synthetic cross. Wheat 4D data are from the F_2 of a Hobbit (contains *Rht-D1b*) × Synthetic cross; segregation for *Rht-D1b* was assayed by seedling responses to gibberellin[12]. Map distances are in centi-Morgans (cM).

fragment length polymorphism mapping showed that C15-1 is tightly linked to *Xpsr1871(Pki)*, a marker that is itself tightly linked with *Rht-D1b* (Fig. 1c). Cereal genomes show substantial conservation in gene order (colinearity). The region of wheat chromosomes 4A, 4B and 4D to which C15-1 hybridizes is colinear with a segment of rice chromosome 3, and with a segment of maize chromosome 1 containing *d8* (Fig. 1c)[17]. These observations are consistent with the hypothesis that the C15-1 transcript is derived from one of the *Rht-1* homoeoalleles.

We used C15-1 to isolate genomic DNA clones containing the putative *Rht-B1a* and *Rht-D1a* (the *Rht-B1* and *Rht-D1* wild-type alleles[5]) and maize *d8* (the *D8* wild-type allele[7]) genes. The amino-acid sequences of the proteins encoded by wheat *Rht-D1a* (Rht-D1a), maize *d8* (d8) and *Arabidopsis GAI* (GAI) and *RGA* (RGA) were compared (Fig. 2a; RGA is an *Arabidopsis* gibberellin signalling protein that is closely related to GAI; ref. 16). Rht-D1a and d8 appear to be more closely related to GAI than they are to RGA (per cent amino-acid identity, GAI versus RGA is, respectively, 62 versus 58% (Rht-D1a) and 62 versus 59% (d8)). The carboxy-terminal ~2/3 of all four proteins are very similar to each other and to the equivalent region of SCARECROW (SCR), a candidate transcription factor from *Arabidopsis*[9,16,18], and of LATERAL SUPPRESSOR (Ls), a tomato protein required for formation of axillary branches during vegetative growth[19]. The N-terminal regions of the GAI/RGA/Rht-D1a/d8 proteins contain two regions of closely related sequence (regions I and II in Fig. 2a). Regions I and II are found in five gibberellin signalling proteins (GAI, RGA, Rht-D1a, d8 and also in Rht-B1a, the *Rht-B1a* gene product; Fig. 3a), but are not found in SCR or Ls, indicating that they may be responsible for the gibberellin-specific action of these proteins[9,16]. Furthermore,

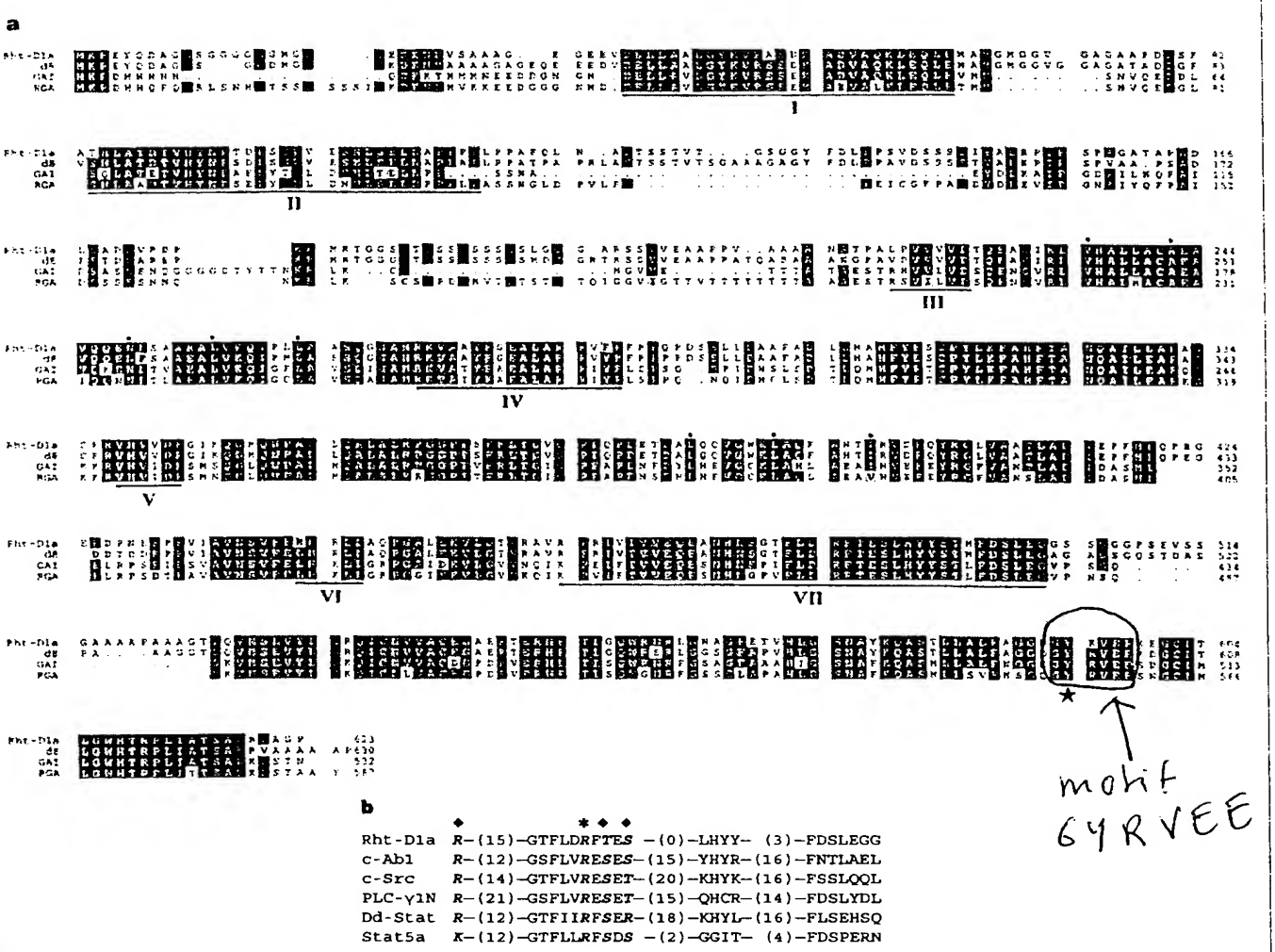

Figure 2 Structural features of Rht-D1a, d8, GAI and RGA. **a**, Amino-acid sequence alignment comparing Rht-D1a, d8, GAI[9] and RGA[16]. Gaps are introduced to maximize alignment. Exact matches are boxed in black, shaded boxes indicate conservative substitutions. Indicated regions of conserved amino-acid sequence are: I and II, N-terminal regions that are not present in SCR[18] or Ls[19]; III and V, valine-rich regions; IV, nuclear-localization signal[9,16]; VI, LXXLL motif[9,16]; VII, SH2-like domain. Residues defining leucine heptad repeats are indicated by closed circles. Star, potential site of tyrosine phosphorylation (this feature, leucine heptad repeats, regions III, V and VII, and the relative positions of all of these features/regions are characteristic of STATs; ref. 22). **b**, Amino-acid sequence alignment comparing sequence from region VII of Rht-D1a (residues 464–503) with those of previously recognized SH2 domains. Functionally significant residues are in bold and italicized. Numbers in parentheses refer to the number of intervening residues that are not shown. A typical SH2 domain is a peptide stretch of 100 amino acids containing an invariant arginine (***R*** with asterisk) that recognizes the phosphate group of phosphotyrosine. Following this arginine are other strongly conserved residues (diamonds). These residues, together with another upstream arginine or lysine (***R*** or ***K*** with diamond), interact with the phosphate group, the tyrosine ring, and the polypeptide backbone of the ligand[20,21]. Other regions of the SH2 domain are less conserved, although their three-dimensional structures are similar. Sequences shown are c-Ab1[10], c-Src[10], PLC-γ1N[10], Dd-Stat[30] and Stat5a[21].

region I is substantially deleted in the *Arabidopsis gai* mutant, confirming that this region is important for gibberellin signalling[9].

Analysis of the GAI/RGA/Rht-D1a/d8 sequences revealed an SH2-like domain within the C-terminal section of the protein (region VII in Fig. 2; Fig. 2b). SH2 domains are associated with phosphotyrosine signalling in metazoans[10], and bind tyrosine-phosphorylated polypeptides at an essential arginine residue. This residue is invariant in SH2 domains and is found in the GAI/RGA/Rht-D1a/d8 SH2-like domain (Fig. 2a, b). Alignment of the Rht-D1a SH2-like domain with previously identified SH2 domains reveals substantial conservation of the amino-acid sequence, especially of those residues that assist in the binding of the phosphorylated tyrosine to the invariant arginine[20,21] (Fig. 2b). To our knowledge, this is the first identified putative SH2 domain in plants. STAT (signal transducers and activators of transcription) proteins are transcriptional regulators that contain SH2 domains[22]. GAI/RGA/Rht-D1a/d8 are candidate transcription factors that contain an SH2-like domain, and display other features characteristic of STATs (for details, see Fig. 2a). Phosphotyrosine signalling may be involved in gibberellin-mediated plant growth regulation, using proteins similar to the STAT factors that mediate cytokine/growth-factor control of growth in animals.

Rht-B1, *Rht-D1* and *d8* are defined by allelic series of semi-dominant mutations that confer differing severities of dwarfism[4–7]. To identify the molecular basis of these mutations, the DNA sequences of five mutant alleles (*Rht-B1b*, *Rht-D1b* and three *D8* alleles) were determined. Each allele contains a mutation that alters the N-terminal region of the protein that it encodes (Fig. 3a). All three maize mutant proteins (D8-1, D8-2023 and D8-Mpl), like *Arabidopsis* gai, lack regions of the peptide sequence. *D8-1* and *D8-2023* are, like *gai*, in-frame deletion mutations. In D8-1, D55 is replaced by a glycine, and 56-VAQK-59 are missing. This segment is very close to that deleted in gai, and falls within the highly conserved region I (Fig. 3a). D8-2023 lacks 87-LATDTVHYNPSD-98 from within the highly conserved region II (Fig. 3a). The *D8-Mpl* mutation is a 330-base pair (bp) deletion that extends from the 5′ untranslated sequence through the presumed (normal) start ATG codon and ends at V84. Genetic analysis[6] indicates that *D8-Mpl*, like *D8-1*, makes an active product. Presumably, *D8-Mpl* translation initiates at M106 (or a subsequent methionine), and makes an N-terminally truncated product that lacks region I and most of region II (Fig. 3a).

The *Rht-B1b* and *Rht-D1b* mutations are both nucleotide substitutions that create stop codons. In *Rht-B1b*, a T-for-C substitution converts the Q64 codon (CGA) to a translational stop codon (TGA; Fig. 3a). In *Rht-D1b*, a T-for-G substitution converts the E61 codon (GGA) to a translational stop codon (TGA; Fig. 3a). The similarity of the *Rht-B1b* and *Rht-D1b* mutations presumably explains why they confer very similar severities of dwarfism[4]. Genetic analysis indicates that both *Rht-B1b* and *Rht-D1b* make active products[12]. It is possible that the short N-terminal peptide fragments encoded by *Rht-B1b* and *Rht-D1b* confer the mutant phenotype. However, it is also possible that ribosomal scanning following translational termination at the mutant stop codons in *Rht-B1b* and *Rht-D1b* permits translational reinitiation at one or other of the several methionines that closely follow these stop codons[23], and that the resultant N-terminally truncated product confers the mutant phenotype. This seems more likely, as the *D8-Mpl* allele also encodes an N-terminally truncated product (see above). Thus *Rht-B1b* and *Rht-D1b*, like *D8-Mpl*, apparently encode N-terminally truncated products that lack region I (Fig. 3a).

Mutagenesis of *Arabidopsis gai* can generate apparent loss-of-function derivative alleles which confer a tall, rather than dwarf, phenotype[24]. These derivative alleles carry mutations that interrupt the *gai* open reading frame (ORF)[9] and thereby abolish *gai* function. Similarly, following fast-neutron mutagenesis, we obtained an apparent loss-of-function allele derived from wheat *Rht-B1b*. This new allele (*Rht-B1g*) confers a tall, gibberellin-responsive phenotype, rather than the dwarf, gibberellin-resistant phenotype characteristic of its *Rht-B1b* progenitor. Gel-blot analysis showed that *Rht-B1g* lacks C15-1-hybridizing DNA derived from chromosome 4B (the chromosome that carries *Rht-B1b*; Fig. 3b), indicating that *Rht-B1g* is a deletion mutation that abolishes *Rht-B1b* function.

The demonstration that, for multiple independent mutant alleles, a heritable change in phenotype is associated with a mutation in a candidate gene is conventionally used as proof that the candidate gene is indeed responsible for the phenotype being studied. Here we have shown that, for three independent mutant *d8* alleles, a heritable change in phenotype (dwarfism, reduced gibberellin response) is associated with a mutation in a candidate *GAI*-like gene. This shows

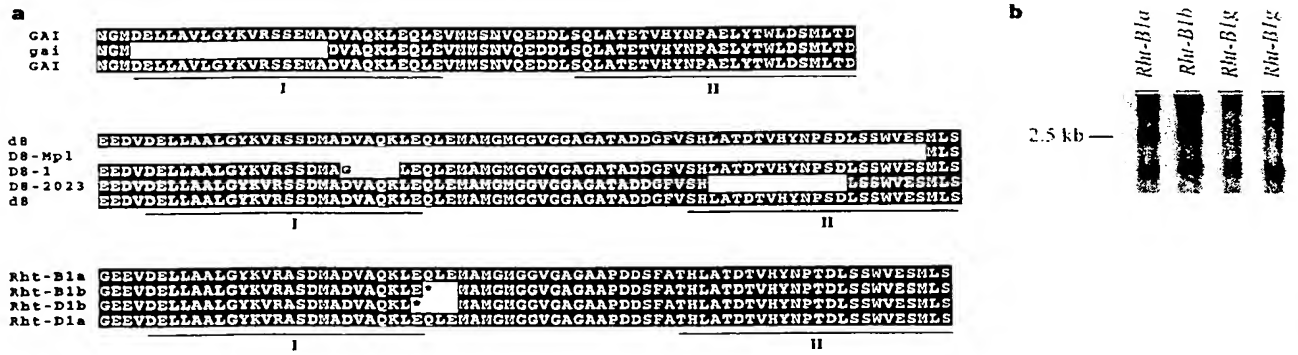

Figure 3 Dominant mutant alleles encode proteins with mutant N termini. **a**, N-terminal segments of predicted proteins encoded by mutant alleles *gai*, *D8-1*, *D8-2023*, *D8-Mpl*, *Rht-B1b* and *Rht-D1b* are compared with those of their respective wild-type alleles (*GAI*, *d8*, *Rht-B1a* and *Rht-D1a*). For each locus, the wild-type sequence is shown above and below the mutant sequence(s). Differences between wild-type and mutant sequences (deletions and substitutions) are highlighted in white, the position of translational stop codons is represented by an asterisk, and the previously identified highly conserved regions I and II (Fig. 2a) are shown. All mutations alter the N-terminal region of their encoded proteins, and affect regions I and/or II. D8-2023 also carries a 6-bp deletion that removes one G and one A residue from 510GAGA513, and a nucleotide substitution that converts T519 to A519 (with respect to d8 sequence; Fig. 2a, and data not shown). Because these altered residues are poorly conserved in GAI/RGA/Rht-D1a/d8, these changes are considered not to be of phenotypic significance. In wheat, Q64 of Rht-B1a is equivalent to Q62 of Rht-D1a, owing to a difference of two amino-acid residues in a poorly conserved N-terminal region (see text; data not shown). *gai* was isolated following X-irradiation mutagenesis[9]; all other mutant alleles shown are of spontaneous origin[4,6,7]. **b**, Gel-blot hybridization of C15-1 with *Bam*HI-digested DNA from *Rht-B1a* (var. Mercia), *Rht-B1b* (Mercia near-isogenic line) and two *Rht-B1g* homozygotes. A hybridizing 2.5-kb *Bam*HI fragment (assigned to chromosome 4B by nullisomic-tetrasomic analysis; data not shown) is missing in the *Rht-B1g* samples.

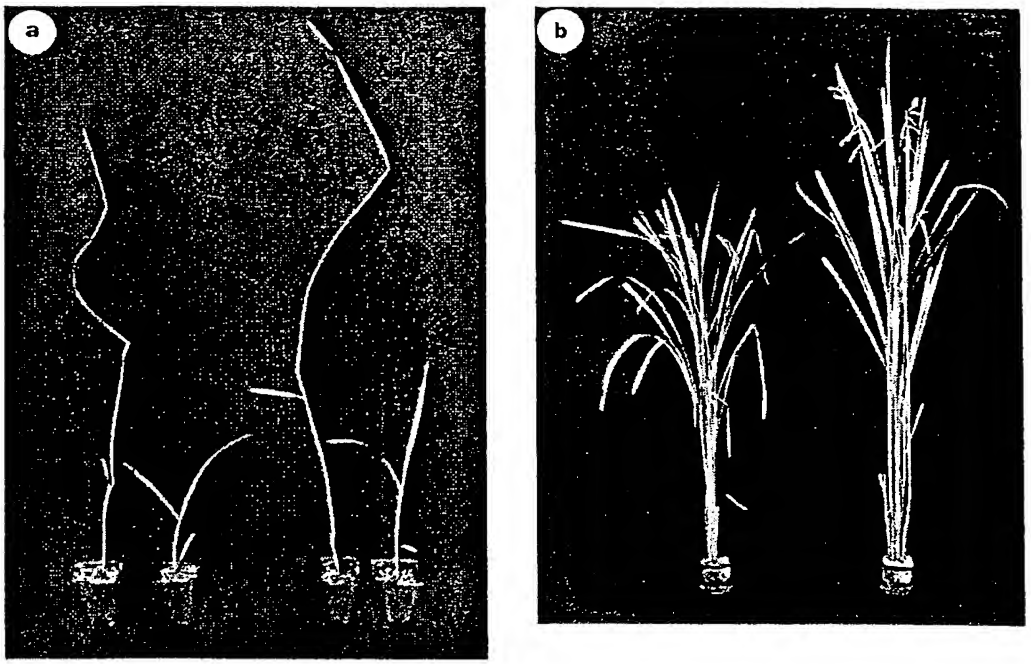

Figure 4 Basmati rice is dwarfed by a construct containing the *gai* ORF. **a**, Primary transformants were allowed to set seed (by self-pollination), and seedling gibberellin-response tests[12] were performed on the progeny. Results from two independent transformant families are shown. Each pair of seedlings consists of two seedlings from the same family: left, a segregant lacking the transgene and displaying the classical elongation response to applied gibberellin; right, a segregant that contains the transgene and is relatively unresponsive to the applied gibberellin. **b**, Adult plant phenotypes. Right, tall plant lacking the transgene is a segregant from the same family as the dwarf plant (left) that contains the transgene.

that we have cloned maize *d8*. The protein encoded by *d8* is closely related to that encoded by the wheat *GAI*-like genes (d8 shows 88% amino-acid identity with Rht-B1a and Rht-D1a), and these genes all map to the same region of the 'ancestral' cereal genome[17]. Thus it is reasonable to assume that maize *d8* and the wheat *GAI*-like genes are the same gene (are orthologues) in these two species. As a whole, our results show that each of five independent dominant mutant alleles (at *d8* and *Rht-1*) is associated with a mutation in this orthologous *GAI*-like gene, demonstrating that we have cloned *Rht-1* and *d8*. The deletion in *Rht-B1g* provides further confirmation that *Rht-B1* is an orthologue of *Arabidopsis GAI*, and that *Rht-B1b* is a mutant allele of this cloned gene.

Height reduction has been associated with yield increases and yield stability in a number of different crop species[3]. Dwarfing mutant alleles of *GAI*, *Rht-1* or *d8* can now be used directly to reduce the height of diverse crops. As a test of this, we introduced constructs expressing the gai protein into Basmati 370 rice (Fig. 4a, b). This rice is commonly grown in northern and north-western regions of the Indian subcontinent. Basmati 370 grain is popular because it is long and slender, is translucent white, cooks well and has a pleasant aroma. However, the plants are tall, with weak culms (stems), and are highly susceptible to damage by wind and rain. This damage causes considerable yield losses and a reduction in grain quality. Previous attempts (using conventional breeding methods) to reduce the height of Basmati 370 while retaining its good qualities were not successful owing to loss of the unique characters for which it is valued. In our experiments, seedling segregants carrying the gai-expressing construct exhibited reduced responses to gibberellin, whereas segregants lacking the transgene responded normally to gibberellin (Fig. 4a). Adult plants carrying the transgene were dwarfed with respect to control segregants lacking the transgene (Fig. 4b). It is now possible to insert a single, genetically dominant, potentially yield-enhancing, dwarfing gene into the genome of any transformable crop, without the need for long-term conventional breeding programmes and with minimal disruption of genetic background.

Our results show that *Arabidopsis GAI*, wheat *Rht-1* and maize *d8* are functional orthologues. Gibberellin signalling appears to be very similar in monocotyledonous and dicotyledonous plants, and may involve the interaction of an SH2-like domain with a phosphorylated tyrosine residue. The mutations in the dominant dwarfing alleles of *D8* and *Rht-1*, like the mutation in the *gai* allele, affect the N-terminal region of the proteins that they encode. Previously, we proposed that GAI is a growth repressor whose action is opposed by gibberellin, and that gai is a mutant repressor that is relatively insensitive to the effects gibberellin[6,9]. According to this view, our data show that a range of different N-terminal deletions and truncations convert GAI/Rht-B1a/Rht-D1a/d8 into mutant repressors that are less affected by gibberellin than the normal protein. This confirms the importance of this N-terminal region for gibberellin signalling and is also consistent with the 'altered function' mode of dominance exhibited by the dominant mutant alleles of *GAI*, *Rht-1* and *d8*[6,7,9,12]. Gibberellin elicits plant responses in a dose-dependent fashion[15]. The fact that different dominant mutant alleles of *Rht-1* and *d8* confer differing severities of dwarfism[4–7] indicates that one of the functions of GAI/RGA/Rht-B1a/Rht-D1a/d8 may be to modulate the gibberellin dose-response. Different amino-terminal deletions and truncations may differentially alter the magnitude of response to a given gibberellin dose, and the structure of this amino-terminal region may be key to the modulator function of GAI/RGA/Rht-B1a/Rht-D1a/d8. □

Methods

Molecular cloning, DNA gel-blot hybridization and DNA sequencing. We isolated wheat cDNA and genomic DNA and maize genomic DNA clones using low-stringency library screens[25]. Wheat DNA gel-blot hybridizations were performed as described[26]. Wheat genomic DNA clones were assigned to their chromosome of origin (4A, 4B or 4D) by identification of restriction fragments

previously assigned through DNA gel-blot analysis of nullisomic-tetrasomic lines. DNA sequencing was done using the Big Dye terminator cycle sequencing kit (Perkin Elmer). The entire coding sequence of each mutant gene (and of wild-type controls) was amplified from genomic DNA (using primers specific to *Rht-B1*, *Rht-D1* or *d8*, as appropriate) by PCR (GeneAmp XL PCR kit, Perkin Elmer). All wild-type and mutant *Rht-1* alleles were amplified from homozygous material. Amplification products were cloned into the pGEM-T Easy vector (Promega). For each gene, we determined DNA sequences from at least two independent amplifications, thus avoiding potential PCR-induced errors. Genetic analyses confirmed that the mutant *D8-1* and *Rht-D1b* sequences co-segregated with their respective mutant phenotypes. For *D8-1*, PCR analysis of the F_1 progeny of a *D8-1/d8* × *d8/d8* cross revealed five dwarf (*D8-1/d8*) plants that were heterozygous for the deletion mutation associated with *D8-1* (see text) and five tall (*d8/d8*) plants that did not carry this deletion. For *Rht-D1b*, the associated nucleotide substitution (see text) was found in three dwarf (*Rht-D1b/Rht-D1b*) but not in three tall (*Rht-D1a/Rht-D1a*) F_2 progeny of a *Rht-D1a/Rht-D1a* × *Rht-D1b/Rht-D1b* cross.

Isolation of *Rht-B1g*. We irradiated 3,000 wheat seeds (var. Highbury, homozygous for *Rht-B1b*) with 3.0 Gy fast-neutrons. *Rht-B1g* was identified as a tall, gibberellin-responsive[12] segregant in an M_2 family derived from self-pollination of an M_1 plant.

Rice transformants. We generated transgenic rice plants expressing the *Arabidopsis* gai protein by particle-gun-mediated transformation[27] using a construct in which the *gai* ORF was expressed under the control of the maize ubiquitin promoter. Presence of the *gai*-containing transgene was verified by PCR amplification[9]. The progeny (derived from self-pollination) of six independent primary transgenic plants were tested for segregation of the transgene and for gibberellin response[12]. In all six families, the transgene and phenotype were perfectly co-segregated: all plants exhibiting a normal gibberellin response lacked a detectable transgene, and all plants exhibiting a reduced gibberellin response contained the transgene. Thus, the reduced gibberellin response phenotype is due to the transgene, and not to inactivation of genes resulting from insertion of the transgene into the rice genome, or to genetic variation generated by the transformation procedure *per se*. Control transformants containing the vector but lacking *gai* were not dwarfed (data not shown).

Illustrations. The amino-acid sequence alignments in Fig. 2a were done using software from the Wisconsin Package (Genetics Computer Group) with default parameters. Alignments in Fig. 2b were made by eye.

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EMBL data library accession numbers are: AJ242530, AJ242531.

Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1

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The underlying causes of obesity are poorly understood but probably involve complex interactions between many neurotransmitter and neuropeptide systems involved in the regulation of food intake and energy balance. Three pieces of evidence indicate that the neuropeptide melanin-concentrating hormone (MCH) is an important component of this system. First, MCH stimulates feeding when injected directly into rat brains[1,2]; second, the messenger RNA for the MCH precursor is upregulated in the hypothalamus of genetically obese mice and in fasted animals[1]; and third, mice lacking MCH eat less and are lean[3]. MCH antagonists might, therefore, provide a treatment for obesity. However, the development of such molecules has been hampered because the identity of the MCH receptor has been unknown until now. Here we show that the 353-amino-acid human orphan G-protein-coupled receptor SLC-1 (ref. 4) expressed in HEK293 cells binds MCH with sub-nanomolar affinity, and is stimulated by MCH to mobilize intracellular Ca^{2+} and reduce forskolin-elevated cyclic AMP levels. We also show that SLC-1 messenger RNA and protein is expressed in the ventromedial and dorsomedial nuclei of the hypothalamus, consistent with a role for SLC-1 in mediating the effects of MCH on feeding.

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The Arabidopsis *RGA* Gene Encodes a Transcriptional Regulator Repressing the Gibberellin Signal Transduction Pathway

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The recessive *rga* mutation is able to partially suppress phenotypic defects of the Arabidopsis gibberellin (GA) biosynthetic mutant *ga1-3*. Defects in stem elongation, flowering time, and leaf abaxial trichome initiation are suppressed by *rga*. This indicates that *RGA* is a negative regulator of the GA signal transduction pathway. We have identified 10 additional alleles of *rga* from a fast-neutron mutagenized *ga1-3* population and used them to isolate the *RGA* gene by genomic subtraction. Our data suggest that RGA may be functioning as a transcriptional regulator. *RGA* was found to be a member of the VHIID regulatory family, which includes the radial root organizing gene *SCARECROW* and another GA signal transduction repressor, *GAI*. RGA and GAI proteins share a high degree of homology, but their N termini are more divergent. The presence of several structural features, including homopolymeric serine and threonine residues, a putative nuclear localization signal, leucine heptad repeats, and an LXXLL motif, indicates that the RGA protein may be a transcriptional regulator that represses the GA response. In support of the putative nuclear localization signal, we demonstrated that a transiently expressed green fluorescent protein–RGA fusion protein is localized to the nucleus in onion epidermal cells. Because the *rga* mutation abolished the high level of expression of the GA biosynthetic gene *GA4* in the *ga1-3* mutant background, we conclude that *RGA* may also play a role in controlling GA biosynthesis.

INTRODUCTION

Gibberellins (GAs) comprise a large family of diterpenoid compounds. Some of these are bioactive plant hormones controlling diverse growth and developmental processes, including seed germination, stem elongation, and flower development (Davies, 1995). Despite its complexity, the GA biosynthetic pathway has been well characterized by using biochemical techniques as well as studying mutants defective in biosynthesis. (GA biosynthesis is reviewed in Hedden and Kamiya [1997].) In contrast, much less is known about how plants perceive GA and how the signal is transduced to control GA-regulated gene expression during plant growth and development. Biochemical studies using barley aleurone cells have demonstrated that GA is perceived on the external face of the plasma membrane (Hooley et al., 1991; Gilroy and Jones, 1994). However, the GA receptor has not yet been identified.

Genetic approaches have been successful in identifying GA signal transduction mutants from a variety of species (reviewed in Hooley, 1994; Ross, 1994; Swain and Olszewski, 1996; Ross et al., 1997). GA response mutants fall into two phenotypic categories: elongated slender mutants and GA-unresponsive dwarf mutants. The recessive slender mutants behave as though their GA response pathway is constitutively activated; they can be further subdivided into GA-responsive and GA-unresponsive mutants. In contrast, the GA-unresponsive dwarfs are semidominant mutants whose phenotype resembles GA-deficient biosynthetic mutants. However, their dwarf phenotype cannot be rescued by exogenous GA treatment. Therefore, these mutants appear to be impaired in GA perception or signal transduction. Unfortunately, most of these mutants are of species not amenable to facile map-based cloning and genetic manipulation. In Arabidopsis, the GA-responsive recessive slender mutant *spindly* (*spy*; Jacobsen and Olszewski, 1993; Jacobsen et al., 1996; Silverstone et al., 1997b) and semidominant semidwarf mutant *gai*, whose stem growth is unresponsive to exogenous GA treatment (Koornneef et al., 1985; Peng and Harberd, 1993; Wilson and Somerville, 1995), have been characterized in detail. Because *spy* alleles are recessive, the *SPY* locus has been postulated to encode a negative regulator of GA response (Jacobsen and Olszewski, 1993). On the other hand, because *gai* is semidominant and the loss-of-function intragenic *gai* suppressors confer a wild-type phenotype, *GAI* was originally thought to be a redundant activator of the GA response pathway (Peng and Harberd, 1993). However, further characterization of a null *gai* mutant (*gai-t6*), using paclobutrazol, an inhibitor of GA biosynthetic

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enzymes, demonstrates that this mutant is more resistant to paclobutrazol than is the wild type (Peng et al., 1997). This result indicates that *GAI* may in fact also be a negative regulator of the GA response. Because *spy* is epistatic to *gai*, it was proposed that *spy* is downstream of *gai* on the GA signal transduction pathway (Jacobsen et al., 1996).

Recently, we identified a new Arabidopsis locus, *RGA* (for repressor of the *ga1-3* mutant), involved in GA response (Silverstone et al., 1997b). Mutant alleles at this locus were isolated as recessive suppressors of the GA biosynthetic mutant *ga1-3*, which is a nongerminating, male-sterile, extreme dwarf blocked in the first committed step of GA biosynthesis (Koornneef and Van der Veen, 1980; Sun and Kamiya, 1994). Mutations at the *RGA* locus partially suppress certain aspects of the GA-deficient phenotype of the *ga1-3* mutant, including the defects in stem elongation, flowering time, and leaf abaxial trichome initiation (Silverstone et al., 1997b). These results suggest that the wild-type RGA protein may function as a negative regulator of the response to GA. *spy*, on the other hand, is able to partially suppress all aspects of the *ga1* mutant (Jacobsen and Olszewski, 1993; Silverstone et al., 1997b). We recently proposed that the *RGA* and *SPY* loci may control separate branches on the GA signal transduction pathway based on epistatic analyses showing that the *rga* and *spy* mutations have an additive effect in the *ga1-3* background (Silverstone et al., 1997b). Subsequently, a fourth locus, *PICKLE* (*PKL*), that may be involved in a more specific set of GA responses, was identified based on characterization of the *pkl* mutation that affects GA-induced differentiation of the seedling primary root (Ogas et al., 1997).

Although *SPY* and *GAI* have been cloned, their exact functions are not well understood (Jacobsen et al., 1996; Peng et al., 1997). SPY shows sequence similarity to Ser (Thr)–*O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferases, which play an important role in regulating the activities (via glycosylation) of various nuclear and cytosolic proteins (Kreppel et al., 1997; Lubas et al., 1997). The *GAI* gene encodes a member of the VHIID regulatory protein family and has structural features indicative of a transcriptional regulator (Peng et al., 1997).

To gain more insight into the function of the RGA protein in the GA response, we cloned the *RGA* locus by genomic subtraction. An additional 10 *rga* alleles, *rga-18* through *rga-27*, were isolated from the M_2 generation of a population of *ga1-3* plants mutagenized by using fast-neutron (FN) bombardment. Four arbitrarily chosen FN alleles were analyzed by genomic subtraction, and a DNA fragment deleted in *rga-20* was identified. DNA sequence analyses of the *RGA* gene indicated that RGA is also a member of the newly identified VHIID family of plant regulatory proteins (Di Laurenzio et al., 1996). RNA expression studies showed that the *RGA* gene is ubiquitously expressed in different tissues and may also play a role in regulating GA biosynthesis. Nuclear localization of RGA was illustrated by the location of a green fluorescent protein (GFP)–RGA fusion protein in a transient expression system. The *rga* mutant was identified in the wild-type *GA1* background, and it does not have a dramatic phenotype.

RESULTS

Cloning of the *RGA* Locus by Genomic Subtraction

Previously, we had isolated 17 independent *rga/ga1-3* mutants from ethyl methanesulfonate–mutagenized *ga1-3* seeds (Silverstone et al., 1997b). Our initial mapping of the *RGA* gene indicated that it was far from any known marker (Silverstone et al., 1997b), precluding the use of map-based cloning. To use the genomic subtraction technique (Sun et al., 1992a) in cloning the *RGA* gene, we isolated an additional 10 mutant alleles of *rga* (*rga-18* through *rga-27*) from an FN-mutagenized population of *ga1-3* mutants. FN bombardment of seeds generates DNA rearrangements and large deletions (Koornneef et al., 1982; Shirley et al., 1992; Sun et al., 1992a; Bruggemann et al., 1996; Cutler et al., 1996). Because there had been no quantitative measure of the frequency with which FN causes large deletions in Arabidopsis, we chose four of our FN-induced *rga* alleles (*rga-18* through *rga-21*) randomly and analyzed them by using genomic subtraction. The four alleles were examined by subjecting *ga1-3* DNA to five rounds of subtraction with biotinylated genomic DNA from the respective *rga/ga1-3* mutant. Afterward, the remaining DNA was amplified and cloned into the pBluescript SK+ plasmid. Individual clones were analyzed for a deletion in the *rga/ga1-3* mutant by DNA gel blot analyses.

We identified a 450-bp DNA fragment (in pRG1) that was deleted in *rga-20* but present in the other three alleles analyzed by genomic subtraction. This fragment is also present in *ga1-3* and Landsberg *erecta* (L*er*; Figure 1A). The insert in pRG1 was used as a hybridization probe to isolate overlapping genomic clones pRG2 and pRG3 from a pOCA18 genomic library (Olszewski et al., 1988) (Figure 2). The genomic DNA corresponding to the inserts in pRG2 and pRG3 was completely deleted in *rga-20* (Figures 1 and 2). A DNA gel blot containing HindIII-digested genomic DNA isolated from nine FN *rga/ga1-3* alleles was hybridized with a DNA probe containing the 2.5-kb left distal end of the insert DNA in pRG3. Figure 1B shows that two additional alleles, *rga-24* and *rga-26*, also had at least 3-kb deletions (1- and 2-kb HindIII fragments) in this region. We did not obtain any genomic clones from the pOCA18 library that extended beyond the left distal end of the insert in pRG3, probably because the library used was amplified from a fraction of the original library.

We then screened for additional genomic clones from a λGEM-11 ecotype Columbia (Col-0) Arabidopsis genomic DNA library. A 2-kb HindIII fragment that was cloned from the left end of pRG3 was used to identify three additional

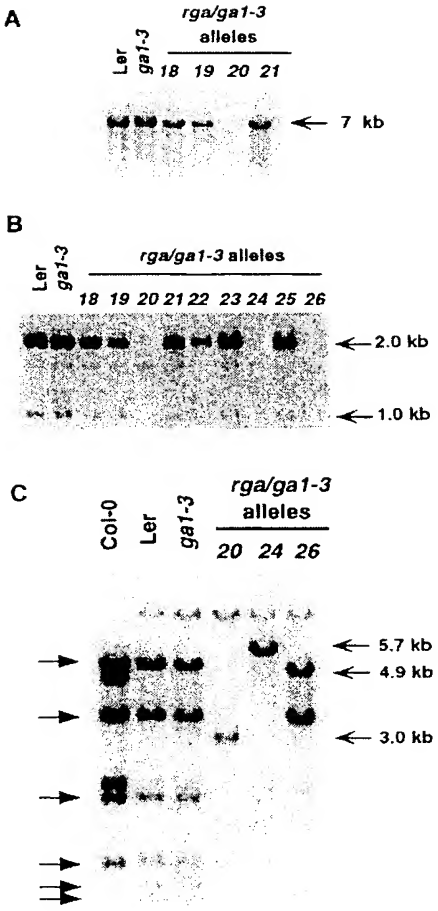

Figure 1. Detection of Deletions in FN-Generated *rga* Alleles.

Shown is autoradiography of DNA gel blots containing HindIII-digested genomic DNA isolated from Col-0, L*er*, *ga1-3*, and FN-generated *rga/ga1-3* mutants. The radiolabeled probes are as indicated.
(A) The 450-bp Sau3A fragment from pRG1.
(B) The 6-kb BamHI-KpnI DNA fragment from pRG3, which includes the 2.5-kb left distal end of the insert DNA.
(C) The AvrII DNA fragment (15 kb) that contains the entire insert DNA of the λRG2 clone
The arrows at right in **(A)** and **(B)** indicate HindIII fragments near the *RGA* locus. The arrows at left in **(C)** indicate HindIII fragments (5, 3.5, 2, 1.4, 1.2, and 1.0 kb, top to bottom) that are absent in the *rga/ga1-3* deletion mutants. The new HindIII fragments present in the deletion mutants are indicated by the arrows at right.

genomic clones: λRG1, λRG2, and λRG3 (Figure 2). These clones were used to map the deleted regions in *rga-24* and *rga-26* as well as the left junction of the deletion in *rga-20* by using DNA gel blot analyses (Figures 1C and 2). *rga-20* has at least a 33-kb deletion, *rga-24* an 8.4-kb deletion, and *rga-26* a 5.9-kb deletion. The 2-kb HindIII DNA fragment, which is completely missing in all three *rga* deletion alleles, was then used as a hybridization probe to identify a putative *RGA* transcript by RNA gel blot analysis. This putative *RGA* mRNA is 2.4 kb and is present in *ga1-3* but absent in *rga-20*, *rga-24*, and *rga-26* (data not shown). Subsequently, we isolated three cDNA clones by screening the λPRL2 Arabidopsis cDNA library with the 2-kb HindIII fragment. The largest clone (pRG20) carries a 2.3-kb DNA insert containing an open reading frame of 1921 bp that encodes a 587-amino acid protein with a 64-kD predicted molecular mass and is likely to be a full-length cDNA because there is a stop codon three nucleotides upstream of the ATG start site.

DNA sequence analysis of the genomic DNA revealed that the *RGA* locus has an uninterrupted 1921-bp open reading frame with no introns. To prove that the cloned pRG20 corresponds to the *RGA* locus, we characterized the molecular

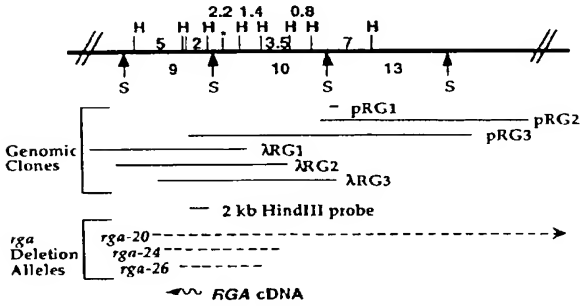

Figure 2. Physical Map around the *RGA* Locus.

The heavy horizontal line shows a SalI (S) and HindIII (H) restriction map around the *RGA* locus. The asterisk indicates a HindIII site that is only present in L*er* but not in Col-0. Distances between restriction sites are indicated in kilobases. The thin horizontal lines labeled Genomic Clones indicate where the original deleted fragment, pRG1, isolated by genomic subtraction, maps in relation to the five overlapping genomic clones. The dashed lines indicate the deleted regions in the three *rga* deletion alleles. The right distal end of the deletion in *rga-20* has not been identified because it is beyond the right distal end of this map. The locations of the left junction of the deletion in *rga-20* and both ends of deletions in *rga-24* are within the HindIII fragment indicated on the map. However, the exact end points have not been determined. The wavy line depicts the coding region of the *RGA* locus. The 2-kb HindIII fragment is located within the deleted regions in all three *rga* deletion alleles and has been used as a hybridization probe in DNA and RNA blot analyses, as described in the text.

lesions in five of the *rga* alleles. DNA gel blot analysis using the radiolabeled 2.3-kb RGA cDNA as a probe indicated that the entire coding region for the *RGA* gene is deleted in *rga-20* and *rga-24* (data not shown). By using DNA sequence analysis, we found that 4.2 kb upstream of the ATG start site and 1.7 kb of the coding region of the *RGA* gene had been deleted in *rga-26* (data not shown). Besides the three FN alleles with large deletions, we also identified single nucleotide changes in two ethyl methanesulfonate alleles. In *rga-1*, the third base in the codon for Trp-521 (TGG) is mutated from G to A, creating a stop codon (TGA) mutation that resulted in a C-terminal truncation; in *rga-2*, there is a missense mutation formed when the first base in the codon for Asp-478 (GAT) is mutated from G to A, which resulted in Asn-478 (AAT) (Figure 3). These results confirm that we have cloned the *RGA* gene.

RGA Is a Member of the VHIID Protein Family

There are several interesting regions in the predicted RGA protein sequence. RGA contains homopolymeric regions of serine and threonine at the N terminus and leucine heptad repeats (Figure 3). RGA also has, beginning at Leu-423, an LHKLL motif, which is identical to the consensus sequence LXXLL (where X stands for amino acid) that was recently demonstrated to mediate the binding of steroid receptor coactivator complexes to nuclear receptors (Heery et al., 1997; Torchia et al., 1997). PSORT analysis (Nakai and Kanehisa, 1992; http://psort.nibb.ac.jp/) indicated a high likelihood of nuclear localization of the RGA protein, and it identified a putative bipartite nuclear localization signal (NLS) beginning at Arg-258. The sequence RKVATYFAELARRIYR fits well with the consensus of bipartite NLSs (Raikhel, 1992). Amino acid sequence comparison between the RGA sequence and those in the database indicated that RGA is a member of the VHIID family of regulatory proteins. *RGA* has some homology to *SCR*, which regulates cellular differentiation in Arabidopsis roots (Di Laurenzio et al., 1996). In their conserved regions, amino acids 176 to 580 in RGA and 245 to 649 in SCR are 38% identical and 44% similar.

While preparing this article, we found that *RGA* was also cloned recently by two other groups. In their search for proteins regulating nitrogen metabolism, Truong et al. (1997) identified two homologous Arabidopsis cDNAs that would complement the yeast *gln3 gdh1* strain, which is affected in the regulation of nitrogen metabolism. They named the cDNAs *RGA1* (GenBank accession number Y11336) and *RGA2* (GenBank accession number Y11337), for restoration of growth on ammonia, and characterized them as VHIID protein family members. By a particularly ironic twist of fate, *RGA1* is identical to *RGA*. Also, Peng et al. (1997) recently cloned the GA signal transduction mutant gene *GAI*. In the course of their study, they also cloned a homologous gene, which they termed *GRS* (for *GAI*-related sequence). However, they only present the sequence data of this gene. After receiving the preprint of the paper by Peng et al. (1997) from N. Harberd, we found that *GAI* is identical to *RGA2* and *GRS* is identical to *RGA* (*RGA1*). Although Truong et al. (1997) used a heterologous system to identify *RGA1* and Peng et al. (1997) only report a *GAI* homologous sequence, these reports did not illustrate the function of RGA in plants. However, we have cloned the *RGA* locus based on its mutant phenotype and have demonstrated clearly *RGA*'s important role in mediating GA signal transduction.

Two other full-length members of the VHIID family in Arabidopsis have recently been identified in contigs at the top of chromosome 4 sequenced by the European Union Arabidopsis Genome Project. The first in contig ATFCA8 (GenBank accession number Z97343) is located at nucleotides 26,164 to 28,937. The second is located in contig ATAP22 (GenBank accession number Z99708) at nucleotides 62,096 to 63,475. Because the deduced amino acid sequences of these two proteins have similar degrees of homology to the first three VHIID proteins, we named the former VHS4 and the latter VHS5 (for VHIID homologous sequence). RGA shows 41% identity and 52% similarity with VHS4 versus 24% identity and 33% similarity with VHS5.

The alignment between RGA, GAI, SCR, VHS4, and VHS5 sequences shown in Figure 3 demonstrates that they all contain the central VHIID conserved region. By comparing RGA with the rest of the VHIID family members, we found two additional conserved motifs besides the VHIID domain (Figures 3 and 4). We have named the one located at the C terminus the RVER domain for the presence of this conserved set of amino acids. At the N terminus, there is the acidic DELLA domain, which is present only in RGA and GAI. Besides these fully sequenced genes, there are a number of partially sequenced expressed sequence tags (ESTs) from various plant species as well as a sequence-tagged site from maize that show homology to RGA and appear to be in the VHIID family. Alignments of their DELLA, VHIID, and RVER domains with those of the completely sequenced proteins are shown in Figures 4A to 4C.

Nuclear Localization of the RGA Protein

Because the predicted RGA amino acid sequence has features that are found in transcription regulators, including a putative NLS, we constructed a cauliflower mosaic virus (CaMV) 35S promoter::*GFP–RGA* gene fusion that could be used in transient assays (Varagona et al., 1992; Haseloff et al., 1997). After biolistic bombardment of an onion epidermal layer with a CaMV 35S::GFP control construct or this reporter construct, the GFP signal from the control was observed in 110 cells to be always in both the cytoplasm and nucleus (Figures 5A and 5B), where it has been shown to accumulate (Haseloff et al., 1997). In contrast, the GFP–RGA fusion protein is located exclusively in the nucleus in 89 cells examined (Figures 5C and 5D), indicating that the RGA sequence targets the fusion protein to the nucleus.

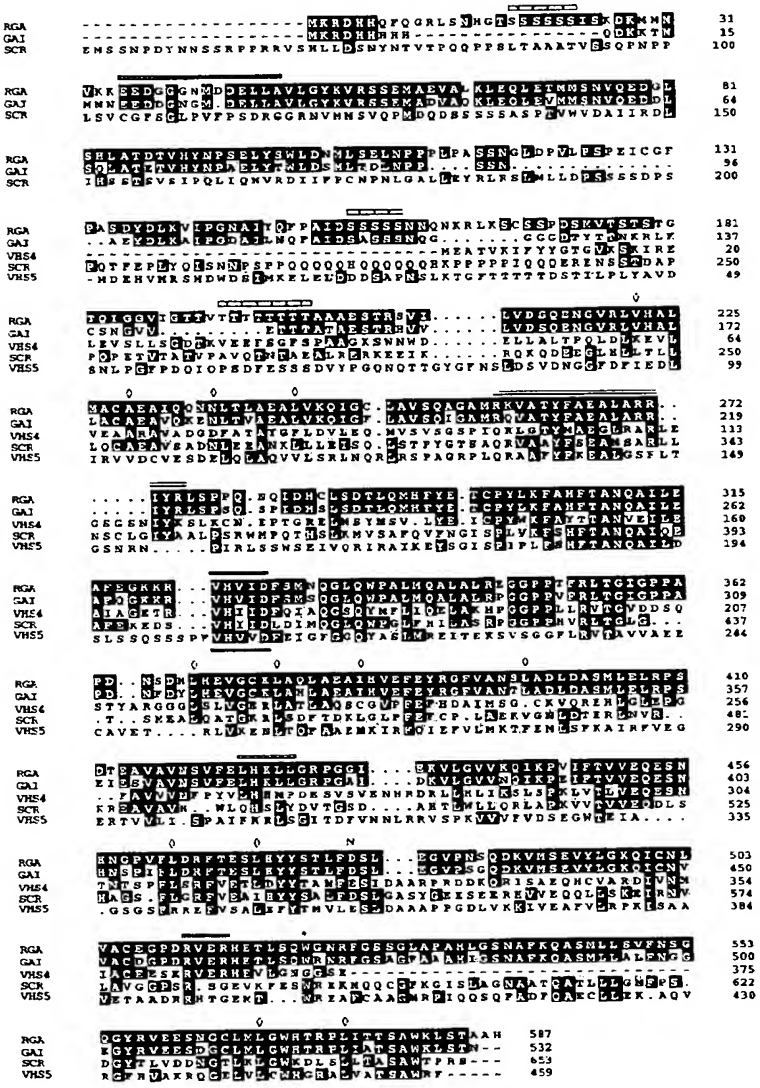

Figure 3. Amino Acid Sequence Alignment of the RGA Protein Compared with Other Members of the VHIID Protein Family.

RGA sequence (L*er* allele) is compared with GAI (Peng et al., 1997; Truong et al., 1997), SCR (Di Laurenzio et al., 1996), VHS4 (GenBank accession number Z97343), and VHS5 (GenBank accession number Z99708). Identical residues conserved between RGA and at least one other family member are displayed in reverse type, and similar residues are in gray boxes. Gaps introduced to improve the alignment are indicated by dots and sequence truncations by wavy dashes. The mutation in *rga-1*, marked above the RGA sequence with an asterisk, changes Trp-520 to a stop codon, and the mutation in *rga-2*, indicated with an N, converts Asp-478 to Asn-478. The acidic DELLA motif is marked by an overhead stippled bar. The conserved VHIID sequence is indicated by thick solid lines above and below the sequences. The RVER motif is marked by an overhead gray bar. The homopolymeric Ser/Thr stretches are indicated by overhead bars with thin stripes. The Leu heptad repeat residues are marked with an open diamond. The putative NLS is indicated by a double line, and the LXXLL motif by a striped bar. The sequence alignment was done using the Pileup program. The boxes were drawn using the BOXSHADE web site (http://ulrec3.unil.ch/software/BOX_form.html).

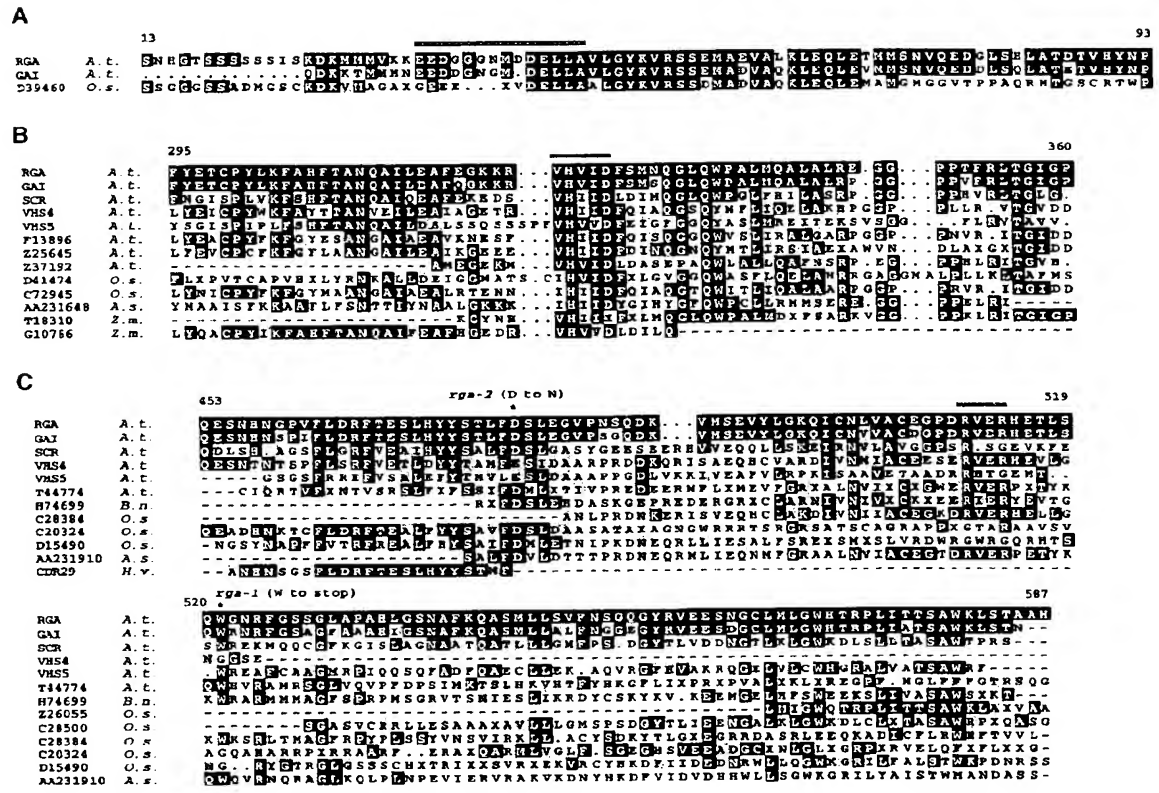

Figure 4. Three Conserved Domains Revealed by Sequence Alignment between RGA, Other Cloned Genes, and ESTs.

Residues conserved between RGA and at least one other family member are displayed in reverse type for identical residues and in gray boxes for similar residues. Gaps introduced to improve the alignment are indicated by dots, and sequence truncations are depicted by wavy dashes. All short sequences are ESTs except for one maize sequence, which is from a sequence-tagged site (G10786). They are labeled according to their GenBank accession numbers. The sequence alignment was done using the Pileup program. The boxes were drawn using the BOXSHADE web site (http://ulrec3.unil.ch/software/BOX_form.html).

(A) The N-terminal DELLA domain.

(B) The central VHIID domain.

(C) The C-terminal RVER domain.

The three motifs are indicated as given in Figure 3. The point mutations in the *rga-1* and *rga-2* mutant alleles are marked above the sequence. *A. t.*, *Arabidopsis thaliana*; *A. s.*, *Avena sativa*; *B. n.*, *Brassica napus*; *H. v.*, *Hordeum vulgare*; *O. s.*, *Oryza sativa*; *Z. m.*, *Zea mays*.

Map Position of the *RGA* Locus

The *RGA* cDNA and genomic DNA clones came from Col-0 libraries. Because our *rga/ga1-3* mutants were all in the L*er* background, we also determined the DNA sequence of the L*er* wild-type allele of *RGA* when we searched for the point mutations in *rga-1* and *rga-2*. Four single-nucleotide polymorphisms were found between the wild-type L*er* and Col-0 alleles of *RGA*. Three are silent changes, but one causes an alteration of the final amino acid residue of the RGA protein (His-587 in L*er* and Tyr-587 in Col-0). This last polymorphism also resulted in the presence of an RsaI site in Col-0 that is absent in L*er*. Although we have previously published a weak linkage for *RGA* at the bottom of chromosome 3 (Silverstone et al., 1997b), we were unable to find any markers that were closely linked to confirm the observation. The RsaI polymorphism between the L*er* and Col-0 alleles allowed us to design a cleaved amplified polymorphic sequence marker that would distinguish between the two ecotypes for mapping by means of the recombinant inbred lines (Lister and Dean, 1993). Using this approach, we found that the *RGA* locus maps very close to the top of chromo-

some 2. This agrees well with the results from Peng et al. (1997), who indicated (as unpublished data) that they had mapped *GRS* to the top of chromosome 2, and Truong et al. (1997), who mapped *RGA1* to three yeast artificial chromosomes that map to the top of chromosome 2.

Identification of the *rga/GA1* Mutant

Previously, we had surmised that *rga/GA1* plants must have a subtle phenotype because we could not identify them in the F_2 generation of a cross between *rga-2/ga1-3* and L*er* (Silverstone et al., 1997b). We subsequently identified *rga-2/GA1*, as described in Methods. These plants were a little paler than wild-type L*er* plants, but they did not have any dramatic phenotype, and they were similar to L*er* with respect to final height, flowering time, and fertility under long-day conditions (data not shown).

Ubiquitous Expression Pattern of *RGA*

To determine whether the regulation of *RGA* gene expression was involved in controlling GA-mediated growth, we measured the levels of the *RGA* mRNA in a number of tissues, including seedlings, roots, rosette leaves, whole rosette plants, bolting stems, mature stems, flower buds, young siliques, and mature siliques (Figures 6 and 7A). We found that *RGA* was expressed ubiquitously in all tissues examined. Quantitative analyses using cyclophilin as a loading control (Lippuner et al., 1994) indicated that the levels of *RGA* mRNA between tissues did not differ greatly (Figure 6).

Previously, we proposed a model of GA signal transduction that consisted of two branches that converge to regulate several common developmental processes, including stem elongation, flowering time, and leaf abaxial trichome initiation (Silverstone et al., 1997b). Our hypothesis was that the plant could achieve finer control over these events by manipulating the signal flowing through the two pathways.

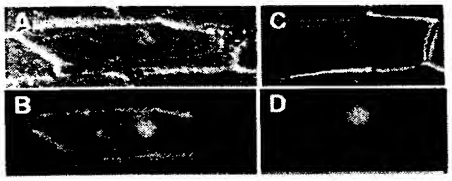

Figure 5. Nuclear Localization of the GFP-RGA protein.

(A) and **(B)** The control GFP protein.
(C) and **(D)** The GFP-RGA fusion protein.
The proteins are transiently expressed in onion epidermal cells. Individual cells are seen in a differential interference contrast image (**[A]** and **[C]**) and a corresponding epifluorescence image (**[B]** and **[D]**), respectively.

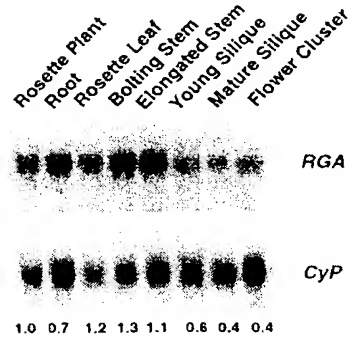

Figure 6. Expression Pattern of the *RGA* Gene

Shown is autoradiography of an RNA blot containing 10 μg of total RNA isolated from different tissues, as labeled. Rosette plants are the 2-week-old aerial portion of the plant, roots are from tissue culture, rosette leaves are from 3 5- to 4-week-old plants, bolting stems are from 2-cm-tall plants (~3 weeks old), elongated stems are from the bottom internode of 3.5- to 4-week-old plants, young siliques were 5 to 7 mm long, mature siliques had fully developed seeds before desiccation, and flower clusters had the terminal inflorescence with developing buds and open flowers. The blot was hybridized with radiolabeled *RGA* cDNA and then reprobed with radiolabeled cyclophilin (*CyP*) as a loading control. The numbers below the blot indicate the relative amount of *RGA* mRNA after standardization, using cyclophilin as a loading control. The level of *RGA* mRNA in the rosette plant was arbitrarily set as 1.0.

Therefore, if one of the branches were constitutively activated, as in the *spy* or *rga* mutant, then the other branch could be inhibited to compensate. This inhibition could occur by altering gene expression of GA response components and/or by modifying their protein activities.

We compared *RGA* expression in wild-type L*er* seedlings to seedlings in a variety of mutant backgrounds, including the GA biosynthetic mutants *ga1-6* (leaky) and *ga1-3* (null) and the signal transduction mutants *rga*, *spy*, and *gai*, both in the wild-type *GA1* background and mutant *ga1-3* background (Figure 7A). Seedlings of these different plant lines grown for 10 days in Murashige and Skoog (MS; Murashige and Skoog, 1962) medium alone were compared with those grown in MS medium containing 1 μM GA_3. Changes in *RGA* expression were quantified by using cyclophilin as a loading control (Figure 7A). Expression of *RGA* was slightly lower in the GA biosynthetic mutants *ga1-3* and *ga1-6* than in wild-type L*er* in the absence of exogenous GA. Except in the *gai* mutant background, there was a slight increase (less than twofold) in *RGA* mRNA levels in all other genetic backgrounds in response to GA application.

Because RGA2 shares 82% identity and 85% similarity with RGA, we thought that *RGA2* may also be involved in GA response. We also examined the *RGA2* gene expression pattern in different GA biosynthetic or signal transduction

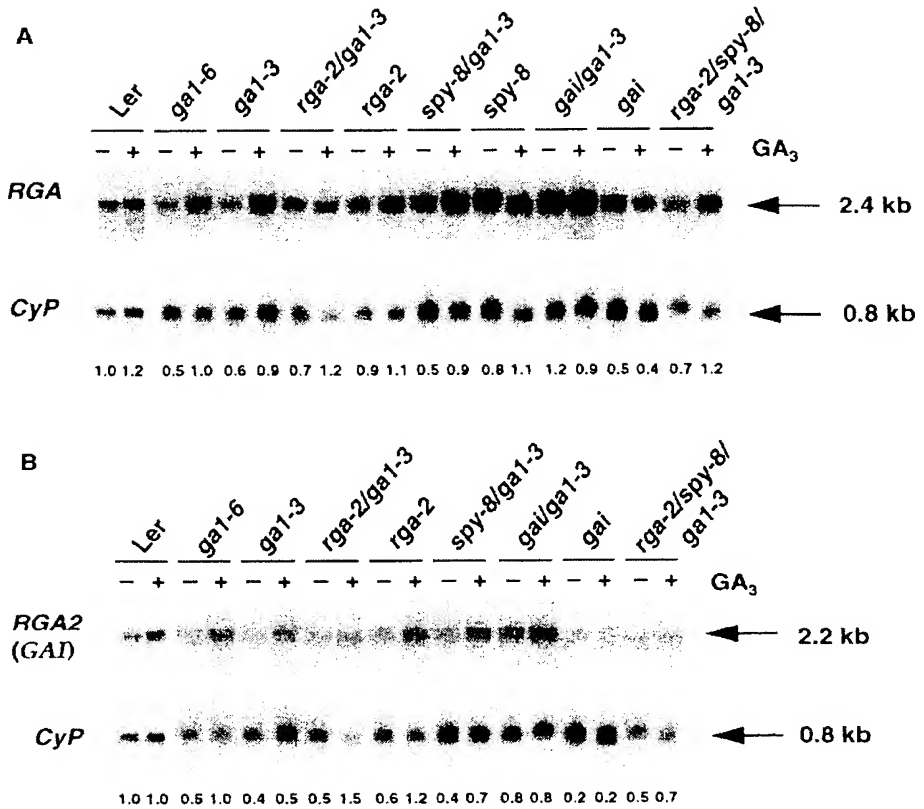

Figure 7. Expression of *RGA* and *RGA2* (*GAI*) in the Wild Type, GA Biosynthetic Mutants, and GA Response Mutants.

Shown is autoradiography of RNA blots containing 10 μg of total RNA isolated from wild-type L*er* and various GA biosynthetic and signal transduction mutants, as labeled. RNA samples were isolated from seedlings grown in media with (+) or without (−) 1 μM GA_3. The arrows at right in **(A)** and **(B)** indicate the sizes of the transcripts.

(A) Blot hybridized with the radiolabeled 2.3-kb *RGA* cDNA and reprobed with cyclophilin (*CyP*). The numbers below each lane indicate the relative amounts of *RGA* mRNA after standardization, using *CyP* as a loading control. The value of L*er* (−GA) was arbitrarily set as 1.0.

(B) Blot probed with a radiolabeled 0.65-kb *RGA2*/*GAI* DNA fragment and reprobed with *CyP*. The relative amount of *RGA2* mRNA is given below each lane, and the value of L*er* (−GA) was set as 1.0.

mutant backgrounds. The pattern of expression we observed for *RGA2* was similar to that of *RGA* (Figure 7B). The difference between expression of the two genes was in the L*er* background, where no increase is seen in *RGA2* expression in response to GA treatment. Now that *RGA2* is known to be *GAI*, the similar expression patterns of these two GA response genes is particularly interesting.

Regulation of GA Biosynthesis

Several of the GA biosynthetic genes have been shown to be under feedback control by GA action, including the GA 20-oxidase genes (Phillips et al., 1995; Xu et al., 1995) and *GA4* that encodes the 3β-hydroxylase, which catalyzes the production of bioactive GAs (Chiang et al., 1995). In the *ga1-3* mutant, which has very low levels of GAs, expression of these genes is elevated, whereas expression in both the *ga1-3* mutant and wild-type plants can be inhibited by GA application. The *gai* mutant is a semidwarf plant blocked in GA signaling, yet it accumulates high levels of GAs (Koornneef et al., 1985). Although GA biosynthesis is upregulated, the *gai* mutant is not able to respond to the increased GA levels. Thus, GA activity has been proposed to modulate GA biosynthesis through feedback inhibition. To determine whether *RGA* is involved in the regulation of GA biosynthesis, we examined

GA4 expression in the different GA biosynthetic and signal transduction mutant backgrounds (Figure 8). *GA4* mRNA level was elevated in the *ga1-3* and *spy/ga1-3* mutants only, but not in the *rga/ga1-3* mutant. In both *ga1-3* and *spy/ga1-3*, the induction of *GA4* expression was inhibited by the application of GA.

DISCUSSION

We have cloned the *RGA* locus by using genomic subtraction. Although the *RGA* sequence is identical to two recently reported genes (Peng et al., 1997; Truong et al., 1997), this study goes beyond these two reports by demonstrating its biological role as a repressor of GA signal transduction. The RGA protein belongs to the VHIID family of regulatory proteins, whose members include SCR and GAI. All three proteins have features indicating that they are transcriptional regulators, and we further showed that the GFP–RGA fusion protein is localized in the nucleus of onion cells in a transient assay (Figure 5). RGA and GAI share a high degree of homology, and both proteins have been suggested to function in GA signal transduction. Based on our analysis of *rga* and *gai* mutant phenotypes (see below) and comparison of the RGA and GAI sequences, we postulate that the two proteins may have overlapping, but not completely redundant, functions in controlling the GA response pathway.

Genomic Subtraction Technique

We have previously shown the utility of the genomic subtraction technique to clone an Arabidopsis gene (Sun et al., 1992a) by using an FN-induced mutant that was likely to contain a large deletion based on genetic fine-structure mapping (Koornneef et al., 1983). In this study, we have demonstrated that the genomic subtraction technique can be used effectively to isolate genes using FN-induced mutant alleles without prior genetic evidence that one or more alleles carry deletions. We found that 33% (three of nine) of our FN *rga* alleles had large deletions affecting the *RGA* gene. In a recent study using FN mutagenesis to identify *hy4* mutants, Bruggemann et al. (1996) found that 15 of 20 mutants contained large (at least 5 kb) deletions. Although the deletion frequency is locus dependent, with a sufficient number of FN alleles (at least four or five), time-consuming fine-structure genetic mapping is not necessary to identify alleles with large deletions before genomic subtraction is performed.

Coding regions in the Arabidopsis genome are very densely organized, with one gene on average every 5 kb (Goodman et al., 1995). It is interesting that the *rga-20/ga1-3* mutant, which has a deletion of at least 33 kb, differs from the other *rga/ga1-3* mutants only in its reduced germination rate, even in the presence of GA. This suggests that no other major genes are likely to be present in this region.

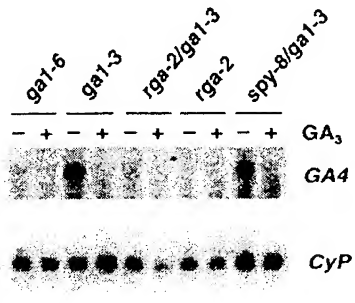

Figure 8. Expression of the *GA4* Gene in GA Biosynthetic and Response Mutant Backgrounds

Shown is autoradiography of RNA blots containing 10 μg of total RNA isolated from various GA biosynthetic and signal transduction mutants, as labeled. RNA samples were isolated from seedlings grown in media with (+) or without (−) 1 μM GA_3. The blot was probed with the radiolabeled 1.4-kb *GA4* cDNA and then reprobed with cyclophilin (*CyP*) as a loading control.

Characterization and Function of the VHIID Family of Regulatory Proteins

Three proteins identified by studies of mutants (RGA, GAI, and SCR) are members of the VHIID family defined by Di Laurenzio et al. (1996). Using the RGA amino acid sequence to search the database with the BLAST program (Altschul et al., 1990), we have identified two additional completely sequenced Arabidopsis genes from the genome project (VHS4 and VHS5), a number of ESTs from Arabidopsis, and ESTs from rice, oat, oilseed rape, and maize with sequence similarity. Three regions of conserved sequence, including an acidic N-terminal DELLA domain, a middle VHIID domain, and a C-terminal RVER domain, have been identified (Figures 4A to 4C). The DELLA domain may be particular to GA response regulatory proteins because it is found only in RGA, GAI, and one rice EST (GenBank accession number D39460). The full-length sequence of this putative rice *RGA* and *GAI* homolog may prove to be interesting. The eponymous VHIID box is more accurately labeled as a (V/I)H(V/I)-(V/I)D box because positions 1, 3, and 4 can be either valine or isoleucine (Figure 4B). Because the RVER domain appears in many VHIID sequences (Figure 4C), this domain may be important for the function of the proteins. So far, the VHIID proteins are found in diverse plant species but not in yeast, prokaryotes, or animals. They are probably ubiquitous in but unique to plants.

A stretch of 23 amino acids at the C-terminal end of RGA shows 78% identity to the N terminus of a barley protein CDR29 (Figure 4C) that is homologous to acyl-CoA oxidases from a variety of species (Grossi et al., 1995). However, the

domain of CDR29 that shares homology to RGA is not in the conserved acyl-CoA oxidase region. *cdr29* expression is induced in barley in response to both dehydration and cold stress (Stanca et al., 1996). Because GA is important in modulating a plant's response to environmental stimuli, this homologous domain may interact with other factors during periods of environmental stress.

The VHIID proteins may be transcriptional regulators. *SCR* has a number of characteristic features, including a putative NLS, homopolymeric Gln, Pro, and Ser, basic leucine zipper, and acidic regions (Di Laurenzio et al., 1996). RGA and GAI have a putative NLS, Leu heptad repeat regions, and the LXXLL motif, and RGA also has homopolymeric Ser and Thr stretches. SCR is proposed to be a transcriptional activator. In contrast, RGA and GAI may be either transcriptional repressors that block transcription of genes involved in GA-regulated growth and development or they may be transcriptional activators that promote expression of such a repressor. In a transient assay using onion epidermal cells, we detected the GFP-RGA fusion protein exclusively in the nucleus (Figure 5). This provides direct evidence that RGA can be properly targeted to the plant cell nucleus. Truong et al. (1997) found that RGA (RGA1) and GAI (RGA2) behaved as transcriptional activators in a heterologous system to allow a yeast *gln3 gdh1* strain to live on ammonia as a nitrogen source. Whether the roles of RGA and GAI in yeast are similar to their roles in plants is not clear. There does not appear to be a yeast homolog of either *RGA* or *GAI*. In addition, the *rga* and *gai* mutant phenotypes do not display any defects in nitrogen metabolism.

Interaction between RGA and GAI

The *gai* mutant was found to have a 17-amino acid in-frame deletion, which may keep the gai protein constitutively active (Peng et al., 1997). This deletion is located within the DELLA domain, which is unique to RGA, GAI, and one rice EST. Consequently, the DELLA domain may be important for GA signal perception or protein deactivation.

Similarity in chemical structure between GA and mammalian steroid hormones has led to the long-standing hypothesis that the two systems shared a similar method of perception and gene regulation. However, there have not been any proteins from plants identified that are homologous to the steroid hormone receptors. The LXXLL motif, recently identified in a number of steroid receptor coactivators (SRCs) and responsible for SRC binding to steroid receptors in the nucleus (Heery et al., 1997; Torchia et al., 1997), is also found in two GA signal transduction components, GAI (Peng et al., 1997) and RGA.

Although *RGA* and *GAI* are very homologous and may share some role in regulating GA signal transduction, they are not completely functionally redundant. Otherwise, the *rga* mutation would not manifest a phenotype in the *ga1-3* background. The N termini of RGA and GAI comprise the most divergent region, suggesting that this region is important for functional differences between the two proteins. Both proteins have leucine heptad repeats that may be involved in protein-protein interactions. Thus, they may form either homodimers or even heterodimers.

In the wild-type background, the *rga* phenotype is subtle, as is the phenotype of the *gai* null mutant *gai-t6* (Peng et al., 1997). The *rga/GA1* plants are a paler green than are L*er* plants, but otherwise flower at the same time, grow to the same height, and have the same fertility. The lack of a dramatic phenotype is discussed further in our model for GA signal transduction, but there is no obvious compensation resulting in increased transcription of one "homolog" in the other mutant background; for example, *GAI* transcription is not affected in the *rga* mutant (Figure 7B). Therefore, if there is any compensation for the loss of one repressor, it would probably occur at the level of translational or post-translational control. Moreover, we did not isolate any *gai* null mutants in our *ga1-3* suppressor mutant screens, even though we did isolate 27 alleles of *rga* and 10 alleles of *spy* (Silverstone et al., 1997b). If *RGA* and *GAI* have similar functions, we would expect *gai* null alleles to suppress partially some aspects of the *ga1-3* phenotype, as *rga* does. Examination of the *gai-t6* mutant in the *ga1-3* background and the *gai-t6/rga* double mutant in both the *ga1-3* and wild-type backgrounds is necessary to determine whether *GAI* has a similar function as *RGA*. If there is any functional redundancy, then we would expect to see some additive effects in the double mutants. Because both proteins seem to be ubiquitous in plants, their activities may be modulated to achieve a fine-tuned response to GA in specific tissues.

The point mutation in *rga-2* (Asp-478 to Asn-478) is in the RVER domain at a highly conserved amino acid in all of the VHIID proteins. This amino acid is an Asp in all proteins except in VHS4 and VHS5 (both have a Glu residue at this position), and this Asp residue is next to a highly conserved Phe residue (Figure 4C). Because *rga-2* is as strong an allele as *rga-1*, which is a nonsense mutation resulting in the C-terminal 67 amino acids being deleted, this Asp residue is likely to play a vital role in VHIID protein function. The three deletion mutants *rga-20/ga1-3*, *rga-24/ga1-3*, and *rga-26/ga1-3* are all phenotypically similar to the other *rga/ga1-3* mutant alleles. Analysis of other point mutations in *rga* alleles may provide additional insights into important functional domains in the RGA protein and possibly in other VHIID proteins.

Because *RGA* and *GAI* are closely related genes and neither has any introns, they may have evolved by a duplication event. Because GAs are found in all seed plants and GA-like compounds are found in ferns and mosses, *RGA* and *GAI* are likely to be part of a conserved signal transduction pathway in plants. Because *RGA* (*RGA1* and *GRS*) and *GAI* (*RGA2*) have been given different names by several groups, for clarity we propose that the names *RGA* and *GAI* be retained for these two genes because the mutant loci had been identified and registered (http://mutant.lse.okstate.edu/genepage/genepage.html) before the cloning of these genes.

Regulation of GA Biosynthesis by RGA

Expression of a 3β-hydroxylase gene (*GA4*; Chiang et al., 1995) is controlled by a feedback mechanism. Although *GA4* expression was increased in the GA-deficient *ga1-3* mutant, it was not detectable in the leaky *ga1-6* missense mutant that is able to germinate, is semidwarf in stature, and is fertile without GA application (Figure 8). This indicates that moderate levels of GAs are able to reduce *GA4* expression. In the *rga/ga1-3* mutant, *GA4* expression was repressed without exogenous GA treatment. Therefore, *RGA* seems to be involved in controlling both GA biosynthesis and GA response. Compared with the *rga/ga1-3* mutant, the *spy/ga1-3* mutant still exhibited a normal *GA4* feedback inhibition response.

Model of GA Signal Transduction

With the cloning of *RGA*, *SPY* (Jacobsen et al., 1996), and *GAI* (Peng et al., 1997), we can present a revised model for GA signal transduction that combines the genetic and biochemical evidence. Our previous model of a branched GA signal transduction pathway was based solely on the genetic data (Silverstone et al., 1997b). We had proposed that one branch is defined by *SPY* and *GAI* and the second branch by *RGA*. These two branches would converge to regulate a common set of developmental processes. The initial cloning of *SPY* did not provide much information about its function, aside from the presence of tetratricopeptide repeats, which mediate protein–protein interactions and occur in a diverse range of proteins (Jacobsen et al., 1996).

However, several Ser (Thr)-*O*-GlcNAc transferases have been cloned recently, and they are homologous to *SPY* (Kreppel et al., 1997; Lubas et al., 1997). These glycosyltransferases can modify proteins by glycosylation alone or by competing for phosphorylation sites. The sites that are modified typically are rich in Ser/Thr, and both RGA and GAI have such a region at their N termini. A second enzyme is required for removing the GlcNAc residue. This raises the possibility that SPY modifies RGA and/or GAI (Peng et al., 1997). SPY could activate these two proteins by transferring a GlcNAc group onto them, and RGA and GAI would then repress genes involved in GA-mediated growth and development. In response to the GA signal, RGA and GAI would no longer have the GlcNAc group, either through competing phosphorylation or simply removal of the GlcNAc residue, and they would not be able to function as repressors. This would explain both the epistasis of the *spy* mutant to the *gai* mutant as well as the additive effects between the *spy* and *rga* mutants. Because they are not functionally redundant, there may be other interacting or modifying proteins that are specific to either RGA or GAI. Activity of these other regulators could explain why *spy* is not epistatic to *rga*.

Peng et al. (1997) provide an elegant model for how GAI functions as a repressor that is turned off directly or indirectly by GAs, thereby allowing growth to occur. In the semidominant *gai* mutant, GAI would be constitutively active and unable to be inactivated, accounting for the dwarf, GA-deficient phenotype. However, because the *gai/ga1* double mutant is an extreme dwarf and can be restored to *gai* semidwarf phenotype by GA application, it is not totally insensitive to GA (Koornneef et al., 1985). At present, the *RGA* and *GAI* homology does not differentiate between whether there are two branches of the signal transduction pathway, with each protein serving a similar role on its respective branch, or whether RGA and GAI actually interact to form a complex that regulates gene expression. In either case, GA would be required to relieve the repression on the pathway, and SPY may be modifying both proteins.

In the *ga1-3* mutant, only a very low level of GA is present, and this is a much more sensitive background in which to observe GA-independent growth. By mutating *rga*, GA signaling is partially de-repressed, and GA-independent stem growth occurs. In the wild-type L*er* plant, there is a higher amount of GA being produced to regulate stem growth. Under these conditions, RGA and GAI may be inactivated by GA directly or indirectly and would only partially repress GA signaling. This may be the reason that a null mutation in either *RGA* or *GAI* does not drastically change the phenotype in the wild-type *GA1* background.

Biochemical studies need to be performed to determine if SPY modifies GAI and/or RGA and to identify other proteins that interact with RGA and GAI. In addition, examination of the genes regulated by RGA and GAI will shed light on the process of GA-mediated growth and development.

METHODS

Plant Materials

Arabidopsis thaliana seeds were stratified for 3 days in the cold before planting. Because *ga1-3* and *rga/ga1-3* mutants require gibberellin (GA) treatment for germination, they were incubated with 100 μM GA_3 during stratification, and the seeds were rinsed thoroughly with water before planting. The plants were grown at 22°C under 16-hr-light/8-hr-dark cycles. For wild-type and mutant seedlings, sterilized and stratified seeds were plated on medium with Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) with or without 1 μM GA_3. After 10 days, whole seedlings were harvested for RNA extraction.

Isolation of Putative *rga* Deletion Mutants

ga1-3 mutant seeds (56,000) were subjected to fast-neutron (FN) bombardment (at the dose 60 Gy) by H. Brunner (Food and Agriculture Organization/International Atomic Energy Agency Agriculture and Biotechnology Laboratory, Vienna, Austria). M_1 plants were grown in flats and allowed to self-pollinate; their seeds were collected in 30 separate pools. We screened 20,000 M_2 plants from each pool for mutants with the *rga/ga1-3* phenotype, as previously described (Silverstone et al., 1997b). Because in our previous screen

all of the plants with the *rga/ga1-3* phenotype were allelic, we assumed all our FN mutants with the same phenotype were also alleles of *rga*. Allelism tests were performed at the same time as the genomic subtraction experiments. Allelism was determined for *rga-20* through *rga-27* by crossing the FN mutants with *rga-2/ga1-3*. The F_1 plants all had the *rga/ga1-3* phenotype

Identification of *rga* in the Wild-Type *GA1* Background

We backcrossed *rga-2/ga1-3* to wild-type Landsberg *erecta* (L*er*) plants. In the F_2 generation, there were no plants with a phenotype differing from L*er*, *ga1-3*, or *rga/ga1-3*. Therefore, we had surmised that if the *rga/GA1* mutant had any phenotype, it would be subtle (Silverstone et al., 1997b). Among the F_2 progeny, we identified wild-type-looking plants that were heterozygous at the *GA1* locus (*GA1/ga1-3*) by using the polymerase chain reaction (PCR) markers described previously (Silverstone et al., 1997b). We then let these individuals self and collected F_3 seeds from each plant individually. The F_3 seeds from each individual were then treated with 100 μM GA_3 for 3 days at 4°C and rinsed thoroughly with water before planting. We could determine the genotype of the original F_2 plant at the *RGA* locus by the following reasoning. If an F_2 plant was homozygous for *RGA*, all of the plants homozygous for *ga1-3* in the F_3 generation would look like *ga1-3*. If an F_2 individual was heterozygous for *RGA/rga-2*, then one-quarter of the F_3 plants homozygous for *ga1-3* would look like *rga/ga1-3* and the rest would look like *ga1-3*. If an F_2 plant was homozygous for *rga-2/rga-2*, then all of the plants homozygous for *ga1-3* would be *rga/ga1-3*. Among the F_3 progeny of an F_2 plant homozygous for *rga-2*, we identified plants by PCR analysis that were also homozygous for *GA1*.

Mapping the *RGA* Locus

From our sequencing data, we found that there was an RsaI restriction endonuclease site polymorphic between L*er* and Columbia (Col-0) in the *RGA* locus (Col-0 at 1759 bp [GTAC, RsaI site], L*er* [GCAC]). Genomic DNA from 30 independent recombinant inbred lines (Lister and Dean, 1993) was amplified using two flanking primers 204 (5′-GTTTAAGCAAGCGAGTATGC-3′) and 211 (5′-TTCGATTCAGTTCGGTTTAG-3′), digested with RsaI, and then fractionated by electrophoresis using a 2.5% agarose gel. Each line was then scored for whether the *RGA* allele was L*er* (a 263-bp fragment) or Col-0 (143- and 120-bp fragments). The data were submitted to the NASC web site (http://nasc.nott.ac.uk/), and *RGA* was mapped to the very top of chromosome 2 close to the telomere (LOD 2.9; log-likelihood = −180.28).

Genomic Subtraction

Genomic subtraction was performed in parallel for *rga-18/ga1-3* through *rga-21/ga1-3* mutants, according to the protocol of Sun et al. (1992a, 1992b), with modifications as noted. The *ga1-3* mutant seedlings grown in sterile MS plates for 2 weeks were used to isolate genomic DNA for subtraction. Plant genomic DNA was purified using a QIAGEN (Valencia, CA) column instead of a CsCl gradient, using a procedure including hexadecyltrimethylammonium bromide and chloroform extraction, as recommended by QIAGEN, with slight modification. We used 3 and 10 g of Arabidopsis tissues for QIAGEN genomic-tip 100/G and 500/G columns, respectively. The DNA was eluted from the column with QF buffer (QIAGEN) preheated to 70°C. Photoactivatable biotin was purchased from Pierce (29987G; Rockford, IL). Four sets of subtractive hybridization reactions, each of which contained one of the four putative deletion *rga/ga1-3* mutant DNAs and the *ga1-3* DNA, were performed. After the fifth cycle of subtraction, the remaining DNA fragments were ligated with Sau3A adapters, amplified by PCR, and cloned into the SmaI site of pBluescript SK+ (Stratagene, La Jolla, CA), as described previously (Sun et al., 1992a). Insert DNA of individual clones was amplified using a primer corresponding to the Sau3A adapters, radiolabeled, and used as hybridization probes for DNA blot analyses. Small genomic DNA gel blots containing HindIII-digested DNA isolated from *ga1-3* and one of the *rga/ga1-3* mutants were used for initial screening of putative clones.

Isolation of *RGA* Genomic and cDNA Clones

Initially, a pOCA18 Col-0 genomic library (Olszewski et al., 1988) was screened with the ^{32}P-labeled random-primed PCR fragment from pRG1, and two overlapping genomic clones were identified as pRG2 and pRG3. A 2-kb HindIII fragment from pRG3 was cloned into the HindIII site of pBluescript SK+ to make plasmid pRG13. To generate additional overlapping genomic clones spanning the deletions, the ^{32}P-labeled random-primed 2-kb HindIII fragment from pRG13 was used to probe a λGEM-11 Col-0 genomic library. An additional three overlapping genomic λ clones were identified as λRG1, λRG2, and λRG3.

A cDNA that corresponds to the deleted region was found by screening the λPRL2 cDNA library obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH) with the ^{32}P-labeled random-primed 2-kb HindIII fragment from pRG13. Four clones were isolated. The plasmids were excised from the phage DNA, according to the protocol supplied (Gibco BRL), by plating phage with DH10B cells on an LB plate with 100 μg/mL of ampicillin and 10 mM $MgCl_2$. Restriction digestion analyses indicated that two clones contained a 2.3-kb cDNA insert, and two others contained truncated cDNAs that are part of the 2.3-kb cDNA. The cDNA clone containing the 2.3-kb insert was designated pRG20 (pZL1 with a 2.3-kb insert cloned at the SalI-NotI sites).

DNA Sequence Analysis

DNA sequencing was performed using a Perkin-Elmer dye terminator cycle system with an ABI (Foster City, CA) 377 PRISM DNA sequencer. Subcloned fragments from pRG20 and λRG2 were used as templates to conduct sequence analyses to determine the *RGA* cDNA and genomic sequence for both strands. Fragments of the *RGA* gene were amplified by PCR from genomic DNA isolated from L*er* and the *rga-1/ga1-3* and *rga-2/ga1-3* mutants to identify point mutations in the *rga-1* and *rga-2* alleles. PCR primers and/or internal primers were used for sequencing reactions. DNA sequence analyses were repeated to confirm the point mutations, using template DNA generated by an independent PCR reaction. Primary sequence analysis was performed with MacVector v3.0 (Oxford Molecular, Campbell, CA). Homology searches were performed in the GenBank database, using the BLAST program (Altschul et al., 1990). Align-

ments were made using the Pileup program in the Genetics Computer Group (Madison, WI) package of programs.

DNA and RNA Gel Blot Analyses

Genomic DNA was isolated from 2-week-old L*er*, *rga/ga1-3*, and *ga1-3* seedlings grown on MS plates, and the mutants in the *ga1-3* background had 1 μM GA_3 included in the plates. The DNA was purified on QIAGEN columns, using the protocol described earlier.

One microgram of HindIII-digested genomic DNA was fractionated on 0.8% agarose gels, transferred to GeneScreen membranes (Du Pont–New England Nuclear), and hybridized with gel-purified ^{32}P-labeled DNA fragments (Ausubel et al., 1990).

Total RNA was isolated from different Arabidopsis tissues (Ausubel et al., 1990; Lashbrook et al., 1994; Silverstone et al., 1997a), and 10 μg of total RNA was treated with glyoxal, fractionated on a 1% agarose gel, transferred to GeneScreen membranes (Sambrook et al., 1989), and hybridized with a random-primed ^{32}P-labeled 2.3-kb SalI-BamHI fragment from pRG20 (Church and Gilbert, 1984). To avoid cross-hybridization between *RGA* and *RGA2* (*GAI*), hybridization was performed at 65°C, using the buffer described in Church and Gilbert (1984), and the filters were washed under high-stringency conditions of 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 1% SDS at 65°C followed by 0.2 × SSC and 0.1% SDS at 65°C for 30 min and 0.1 × SSC at room temperature. After autoradiography, filters were stripped and reprobed with the ^{32}P-labeled 0.8-kb EcoRI fragment from the cyclophilin gene as a loading control (Lippuner et al., 1994). The *RGA2* probe for the RNA blot was made by amplifying L*er* genomic DNA with primers 300 (5′-CTAGATCCGACATTGAAGGA-3′) and 201 (5′-CAGCTAAGCATCCGATTTGC-3′), which specifically amplified a 652-bp fragment from *RGA2* (Truong et al., 1997). Primer 300 has an eight-base mismatch with *RGA*, including the three nucleotides at the 3′ end. Primer 201 sequence is identical to *RGA* and has only a single base mismatch with *RGA2*. If these primers had also amplified a fragment from *RGA*, there would have been an additional 801-bp band. The *GA4* probe was made by random-prime labeling the 1.4-kb EcoRI fragment of the *GA4* cDNA cloned into pBluescript SK+. The RNA and DNA blots were exposed to a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) and quantitated on a PhosphorImager (model 400E; Molecular Dynamics), using Imagequant v4.1 software.

Transient Expression of the GFP–RGA Fusion Protein in Onion Epidermal Cells

The *RGA* cDNA from pRG20 was amplified using primers 216 (5′-AACCAGATCTATGAAGAGAGATCATCACCA-3′; BglII site underlined) and 217 (5′-ATTAAGATCTTCAGTACGCCGCCGTCGAGA-3′; BglII site underlined) and the Expand High Fidelity system (Boehringer Mannheim) to generate a BglII site at both the 5′ and 3′ ends of the *RGA* cDNA. This PCR DNA was digested with BglII and ligated with BglII-digested pRTL2ΔNmGFPS65T to create pRG34F, which contains *GFP-RGA* in-frame fusion under the control of the cauliflower mosaic virus (CaMV) 35S promoter. This *GFP-RGA* fusion encodes a fusion protein with GFP at the N-terminal portion and RGA at the C-terminal portion. The onion epidermal layers were prepared and bombarded, as previously described (Varagona et al., 1992), with tungsten particles (Bio-Rad) coated with the control plasmid DNA, pRTL2ΔNmGFPS65T, or pRG34F. The cells were viewed using a Leica (Heerbrugg, Switzerland) DMRB microscope equipped with a fluorescence module.

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RELATED PROCEEDINGS APPENDIX

None.